An investigation of *L. monocytogenes* in the South African ready-to-eat prepared-fruit industry

by
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Declaration

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This study was approved by the Research Ethics Committee: Biosafety and Environmental Ethics, Stellenbosch University – (#BEE-2020-13176)

Summary

Listeria monocytogenes is a foodborne pathogen responsible for numerous incidences of foodborne-illness and deaths annually. It is a robust environmental pathogen and can survive a wide range of conditions, both within the natural and food processing environment. *L. monocytogenes* abundance in soil and nature make it difficult to control in the agricultural setting and thus require adequate management in factories and retailers. Its management within the food industry, especially the ready-to-eat sector is of importance, as these products pose the greatest risk to consumer health, due to the limited measures that can be taken to eliminate this bacterium prior to consumption. This is especially true for prepared-fruit products, which cannot be heat treated due to the negative effects this has on sensory and quality attributes of the final product. Furthermore, fruit is susceptible to contamination pre-harvest due to contamination from soil and irrigation water, where *L. monocytogenes* thrives as a facultative anaerobic, Gram-positive saprotroph. This requires prepared-fruit producers to prioritise the use of sanitation and disinfection measures, as well as adequately controlling temperatures throughout the supply chain to limit the growth and survival of the organism. This must be complemented with proper monitoring procedures to tailor measures to eradicate potential niches within the food processing environment where this organism can survive as well as ensuring that final products are not contaminated and pose a risk to the consumer.

Better understanding of this organism in South Africa is important in the wake of the largest ever recorded listeriosis outbreak which occurred in 2017/2018, especially in the prepared-fruit industry which has limited understanding of their role in controlling *L. monocytogenes*. This study identified climatic and environmental factors within the agricultural environment that may contribute to the contamination of raw materials used in the production of prepared-fruit products, as well as identified raw materials and areas within the prepared-fruit factory that harboured *L. monocytogenes* through environmental sampling and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) lineage typing. Findings from melon farms in Limpopo and the Eastern Cape indicated that cooler and wetter temperatures contributed to the presence of *Listeria* species in the agricultural environment.

Samples from the in-house *Listeria* monitoring program at a prepared-fruit factory in Gauteng indicated the presence of *L. monocytogenes* (n=49) lineage I and II on raw materials, in the environment and in final products. Sanitisers used for the management of this pathogen were also assessed by interpreting the susceptibility of isolates collected via the Kirby-Bauer disk diffusion method. Chlorine, peracetic acid and a quaternary free sanitiser (QFS) were used, with a repeated

measures analysis of variance (ANOVA). This indicated that the source and lineage of *L. monocytogenes* isolates did not result in significant differences to resistance, furthermore it was found that QFS was the only sanitiser of the three to show efficacy against all isolates collected from the food processing environment. QFS should therefore be used to effectively manage *L. monocytogenes*.

Assessment of common chromogenic media used for the detection of *L. monocytogenes* in environmental swabbing and final product sampling compared three media in their ability to detect and select for *L. monocytogenes*. These media were RAPID'L.mono™ (RLM) (Bio-Rad), *Brilliance Listeria* Agar (BLA) (Oxoid) and Harlequin™ *Listeria* Chromogenic agar (HAR) (Neogen) with comparison based on the bacterium, source, and lineage of *L. monocytogenes*, finding that all agars were comparable in their ability to detect *L. monocytogenes*, however both BLA and RLM supported the growth of contaminating bacteria. This work contributed to better understanding *L. monocytogenes* in the prepared-fruit industry and elucidated better control and monitoring measures that can be used by industry to ensure food safety.

Opsomming

Listeria monocytogenes is ‘n voedselverwante patogeen wat jaarliks verantwoordelik is vir talle gevalle van voedselverwante siektes en sterftes. Dit is ‘n robuurste omgewinspatogeen en kan ‘n wye verskeidenheid toestande oorleef, beide in ‘n natuurlike en ‘n voedselverwerkingsomgewing. Omdat *L. monocytogenes* algemeen in die grond en die natuur voorkom, is dit moeilik om in ‘n landbou-omgewing te beheer en voldoende bestuur in fabrieke en kleinhandelaars is dus noodsaaklik. Die bestuur daarvan binne die voedselbedryf is belangrik, veral in die reg-om-te-eet sektor, aangesien hierdie produkte die grootste risiko inhou vir verbruikersgesondheid omdat daar beperkte maatreëls ingestel kan word om hierdie bakterium voor inname uit te skakel. Dit is veral van toepassing op die voorbereide-vrugte produkte wat nie hitte behandeling kan ondergaan nie as gevolg van die negatiewe effek wat dit op die sensoriese en kwaliteitseienskappe van die finale produk sal hê. Verder is vrugte voor oes vatbaar vir kontaminasie vanuit die grond en besproeiingswater waar *L. monocytogenes* floreer as ‘n fakultatiewe anaërobiese, Gram-positiewe saprotroof.

Dit vereis dat voorbereide-vrugte produsente die gebruik van sanitering en ontsmettingsmaatreëls moet prioritiseer, asook die voldoende beheer van temperatuur regdeur die voorsieningsketting om die groei en oorlewing van die organisme te beperk. Dit moet aangevul word met behoorlike moniteringsprosedures om maatreëls aan te pas om potensiële nisse binne die voedselprosesseringsomgewing, waar hierdie organisme kan oorleef, uit te roei asook om te verseker dat die finale produkte nie gekontamineer word en ‘n risiko vir die verbruiker inhou nie. Beter begrip van hierdie organisme in Suid-Afrika is belangrik na aanleiding van die grootste aangetekende listeriose-uitbraak ooit wat in 2017/2018 plaasgevind het, veral in die voorbereide-vrugte bedryf wat ‘n beperkte begrip het van hul rol in die beheer van *L. monocytogenes.* Hierdie studie het klimaats- en omgewingsfaktore binne die landbou-omgewing geïdentifiseer wat kan bydra tot die kontaminering van rou materiale wat gebruik word in die produksie van voorbereide-vrugte produkte, sowel as geïdentifiseerde rou materiale en gebiede binne die voorbereide-vrugte fabriek waar *L. monocytogenes* voorgekom het, deur omgewing-steekproefneming en PCR-RFLP geslagslyn tipering. Bevindinge van spanspekplase in Limpopo en die Oos-Kaap het aangedui dat koeler en natter temperature bygedra het tot die voorkoms van *Listeria* species in die landbou-omgewing. Monsters van die interne *Listeria* moniteringsprogram by ‘n voorbereide-vrugte fabriek in Gauteng het die teenwoordigheid van *L. monocytogenes* (n=49) geslagslyn I en II aangetoon op rou materiale, in die omgewing en in die finale produkte. Saniteerders wat gebruik is vir die beheer van hierdie patogeen is ook geassesseer deur die vatbaarheid van isolate te interpreteer wat versamel is via die Kirby-Bauer

skyf diffusie metode. Chloor, perasynsuur en ‘n kwaternêre-vrye saniteerder (QFS) is gebruik, met ‘n herhaalde variansie-analise (ANOVA), wat aangedui het dat die bron en geslagslyn van *L. monocytogenes* isolate nie ‘n beduidende verskil tot weerstandbiedendheid tot gevolg gehad het nie; verder is daar gevind dat QFS die enigste van die drie saniteerders was wat effektiwiteit getoon het teen al die isolate wat versamel is uit die voedselprosesseringsomgewing en dus gebruik moet word om *L. monocytogenes* effektief te beheer. Assessering van algemene chromogeniese media wat gebruik word vir die opsporing van *L. monocytogenes* in omgewings-deppers en finale produk, het RAPID’L.mono™ (RLM) (Bio-Rad), *Brilliance Listeria* Agar (BLA) (Oxoid) en Harlequin™ *Listeria* Chromogeniese agar (HAR) (Neogen) vergelyk met ‘n algemene kontaminerende bakterium, bron en geslagslyn van isolaat en het gevind dat alle agars vergelykbaar was, maar beide BLA en RLM het die groei van die kontaminerende bakterium ondersteun. Hierdie werk het bygedra tot ‘n beter begrip van *L. monocytogenes* in die voorbereide vrugte bedryf en het lig gewerp op beter beheer- en moniteringsmaatreëls wat deur die industrie gebruik kan word om voedselveiligheid te verseker.

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“The wise man is not the man who gives the right answers; he is the one who asks the right questions.” – Claude Levi-Strauss

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ABC	ATP-binding cassette
ALOA	Agar *Listeria* according to Ottaviani & Agosti
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Benzalkonium chloride
BHI	Brain heart infusion (agar)
BLA	*Brilliance Listeria* agar
Bp	Base pairs
BPW	Buffered peptone water
CAMP	Christie-Atkins-Munch-Peterson
CDC	Centre for Disease Control
CFU	Colony forming unit
CNS	Central nervous system
DNA	Deoxyribonucleic acid
E	Environment
EC	Eastern Cape
ESEM	Environmental scanning electron microscopy
ETP	Easy-to-prepare
FBO	Food business operator
FCS	Food contact surface
FDA	Food and drug administration
FP	Final product
GMP	Good manufacturing procedure

HACCP	Hazard analysis and critical control points
HAR	Harlequin™ *Listeria* Chromogenic agar
HIV/AIDS	Human immunodeficiency virus/Acquired immune deficiency syndrome
HPP	High pressure processing
ISO	International Organization for Standardization
L	Limpopo
LEB	*Listeria* enrichment broth
LiCl	Lithium chloride
LIPI	*Listeria* pathogenicity island
LLO	Listeriolysin O
LMG	Laboratory of Microbiology, Ghent
LPM	Lithium chloride phenylethanol moxalactam agar
LRB	*Listeria* repair broth
LSD	Least significant difference
LSM	Least square mean
MIC	Minimum inhibitory concentration
NGFIS	Netherlands Government Food Inspection Service
NMKL	Nordic Committee on Food Analysis
OCLA	Oxoid chromogenic *Listeria* agar
PAA	Peracetic acid
PALCAM	Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol agar
PC-PLC	Phosphatidylcholine phospholipase C
PCR	Polymerase chain reaction
PFF	Prepared-fruit factory
PFGE	Pulsed-field gel electrophoresis
PFSC	Prepared-fruit supply chain

PI-PLC	Phosphatidylinositol phospholipase C
PVC	Polyvinylchloride
QAC	Quaternary ammonium compound
QFS	Quaternary free sanitiser
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RLM	RAPID'L.mono™ media
RM	Raw material
RNA	Ribonucleic acid
RTE	Ready-to-eat
SANAS	South African National Accreditation System
SHS	Superheated steam
SNP	Single nucleotide polymorphism
ST	Serotype
TAE	Tris base, acetic acid, EDTA
TSA	Tryptic soy agar
UK	United Kingdom
UV	Ultraviolet
UVM	University of Vermont Medium
VBNC	Viable but nonculturable
WGS	Whole genome sequencing
WHO	World Health Organisation

Chapter 1: Introduction

1.1. Introduction

Foodborne illnesses continue to impact the lives of millions of people annually, some suffering short-term symptoms while others may face life-threatening complications and death (Ooi and Lorber, 2005; CDC, 2017; Chen *et al.*, 2020). The role *Listeria monocytogenes* plays in the infection of consumers and proliferation in the food processing facility is made so significant because of the high mortality associated with listeriosis, the disease caused by the organism (Mwamakamba *et al.*, 2012; Olanya *et al.*, 2019). Listeriosis has a reported mortality rate of nearly 30% (Bill *et al.*, 2014), and even though the number of cases reported is significantly less than other foodborne diseases, it is still responsible for a vast proportion of the deaths caused as a result of the consumption of contaminated foods. Most notably the listeriosis outbreak in South Africa during 2017/2018, which resulted in the highest number of mortalities ever recorded as a result of an outbreak, resulting in the death of 204 individuals (Dramowski *et al.*, 2018; Olanya *et al.*, 2019; Witbooi *et al.*, 2020).

The immunocompromised, like the elderly and pregnant women, are at significant risk of contracting listeriosis compared to healthy individuals. Listeriosis in the healthy manifests as self-limiting gastroenteritis, however, in susceptible populations it can result in septicaemia, meningitis and stillbirth or sudden abortion in pregnant women (Batt, 2014). One of the primary vectors for the pathogen is through food products, specifically ready-to-eat (RTE) products which require little to no further processing from the consumer (Contini *et al.*, 2016). This shifts the burden of ensuring food safety primarily to the processor (Orsi *et al.*, 2011).

In this study, an investigation of the occurrence of *L. monocytogenes* was conducted in the South African RTE prepared-fruit industry. Swabbing took place at melon farms in the Eastern Cape and Limpopo as well as a fruit processing facility in Gauteng. The management systems were in place for the monitoring and control of *Listeria* at the site of RTE prepared-fruit production and the incidence of *L. monocytogenes* on raw materials warranted an investigation of this organism at farms producing melons.

Monitoring of *Listeria* spp. in the food industry is a common tactic employed in order to better track, isolate and predict *L. monocytogenes* within the processing environment (Orsi and Wiedmann, 2016). The robust survivability and ubiquity of the entire genus results in collective survivability throughout the natural and processed environment (Orsi and Wiedmann, 2016). Although *L. monocytogenes* is the major human foodborne pathogen within the genus, other species have also been responsible for causing listeriosis, most notably *L. ivanovii* which is typically implicated in causing listeriosis in cattle and other livestock (Linke *et al.*, 2014; McAuley *et al.*, 2014). In some isolated cases there has also been evidence of *L. innocua* meningitis (Fontana *et al.*, 2014). This highlights the need for better understanding of the pathogenicity of the entire genus.

The colonisation of a production facility by *Listeria* spp. is common in the food industry and can generally be deemed inevitable, as the organism is ubiquitous and can survive in several niches within the natural environment (Weis and Seeliger, 1975; Gandhi and Chikindas, 2007; Orsi and Wiedmann, 2016). In a fresh produce processing environment, it can therefore be expected that colonisation of the processing environment by *Listeria* spp. is most likely because of contaminated incoming raw materials, machinery, clothing or staff.

Fruit which have been implicated in listeriosis outbreaks include melons, stone-fruit and caramel apples (McCollum *et al.*, 2013; Chen *et al.*, 2016; Angelo *et al.*, 2017). Research has centred on melons, due to the occurrence of outbreaks in the USA in 2011 and in Australia in 2018, as well as the likelihood of contamination from *L. monocytogenes* due to the close proximity in which they grow to the soil (McCollum *et al.*, 2013; Das, 2019). Other fruit, like papaya, avocado and watermelon, have also been investigated regarding their ability to support the growth of *L. monocytogenes* with findings indicating that these items may also act as vectors for this pathogen (Ukuku and Fett, 2002; Penteado and Leitão, 2004; Feng *et al.*, 2015; Ziegler *et al.*, 2018; Salazar *et al.*, 2020). There is unfortunately limited understanding of *L. monocytogenes*.

Raw materials role in introducing *Listeria* into the production environment is unknown. Due to the production practices employed with fruit there are several factors which increase the risk of *Listeria* contamination like washing, brushing and submersion in dipping in tanks.

The importance of managing a preventable illness is paramount to ensuring the health of consumers, especially in the South African context. Within South Africa there are a number of compounding socio-economic issues which increase the risk associated with listeriosis. The

focus of this thesis is to investigate the presence of *L. monocytogenes* and other *Listeria* spp. at the site of raw material production and the site of processing; the response of isolates from these environments to common sanitisers used in the food industry and the ability of modern chromogenic media to accurately culture and detect *L. monocytogenes*.

Within this research project some of the overarching goals are:

I – To increase the awareness and postulate solutions for the control of *Listeria* spp., particularly *L. monocytogenes*, in the prepared-fruit industry.

II – To highlight the importance of proper *Listeria* management procedures in the prepared-fruit industry with sanitisers.

III – To understand the differences in chromogenic media used for the detection of *L. monocytogenes* and the influence of contaminating bacteria.

IIII – To contribute knowledge to the understanding of *L. monocytogenes* in the fruit industry.

Within South Africa there is limited research on *L. monocytogenes*, specifically with relation to the fruit industry and although there has been increasing interest from institutions and industry, the gap in understanding is still in need of significant bridging. Cantaloupe remains one of the noteworthy fruits with the potential for contamination by *L. monocytogenes*. It has been implicated in listeriosis outbreaks in the USA, as well as in Australia (McCollum *et al.*, 2013; Tambo *et al.*, 2018; Das, 2019). Furthermore, to the author's knowledge, there is little to no research projects which have mapped and analysed the link between *Listeria* spp. at ready-to-eat prepared-fruit processing facilities and the site of raw material production, although research has previously been conducted on whole avocado packing and processing facilities (Strydom *et al.*, 2013, 2016) and in the retail sector (Christison *et al.*, 2008). The outcomes from this thesis can have insightful findings for both growers and processors as this may affect several management procedures and the standards to which producers are held. The means by which proliferation of the organism occurs is also specific to the processing facility, and with scientific investigation there is potential to uncover valuable information which may prove beneficial to stakeholders and the industry.

For this research project it is significant to outline the scope of investigation as there are limitations naturally occurring due to the industrial setting. The fruit processing facility tests for all *Listeria* spp. within their environment, raw materials and finished RTE product. In the results of their testing for 2018/2019 are a significant number of instances of *L. monocytogenes*. *L. monocytogenes* poses a risk as a foodborne pathogen; hence this strain of *Listeria* will be a

primary focus of this investigation. *L. monocytogenes* is itself divided into four distinct groups based on both phylogenetic and subtyping studies. Only lineages I and II will be included due to their prevalence in food environments and clinical cases of listeriosis (Orsi *et al.*, 2011). The factory in question processes several different fruits, the total number varies based on seasonal changes, however the only raw material to be investigated in the agricultural setting will be cantaloupe and watermelon. The primary reason for these two fruits is the ease with which the sites of production can be reached for both fruit and motivation from the stakeholders to investigate these raw materials. There is also a general lack of *Listeria*-related research in the South African context except a study into retail fruit salads (Christison *et al.*, 2008). Both melons are also grown in two different locations within South Africa based on the time of year – October to January is sourced from Limpopo while February to June is from the Eastern Cape – allowing for results to be compared and investigated with geography and weather as a principal variables (Al-Ghazali and Al-Azawi, 1988; Ivanek *et al.*, 2007; Linke *et al.*, 2014; Chersich *et al.*, 2018). Other fruit were excluded from agricultural sampling in this research project due to time constraints, feasibility and level of previous research conducted.

As many of the technical aspects as possible will be equivalent to what is used in industry specifically with regard to swabbing and culturing of *Listeria* spp., the use of sanitisers and chromogenic media. This allows for the results to be interpreted in a way that can contribute meaningfully to the management of *L. monocytogenes* in the prepared-fruit factory and RTE products.

Sampling of the fruit will include the outer surface as well as an internal swab along the vertical axis of the fruit from the stem wound. Sampling within the factory is specific to areas which have either previously been identified as *Listeria* harbourage sites, are not included in the in-house *Listeria* management programme or are considered high-risk by technical and managerial staff at the processing facility. High-risk areas will be determined at the site of production based on previous evidence of "hot-spots". It must be noted that no part of the food supply chain post-packaging will be investigated due to accessibility, time, and feasibility.

Lastly, a major limitation is that the factory is dynamic and placement of certain equipment and procedures changes both seasonally and depending on factory maintenance and other operational requirements. As a result, microbial colonies may be subject to elimination or dissemination. This may result in inconsistencies or outlying data which must be interpreted, keeping in mind the dynamic nature of the processing environment.

Some assumptions have been made in the undertaking of this research. One assumption is that the staff at the processing facility follow protocols for the management of food safety outlined in their own hazard analysis and critical control points (HACCP) plan as well those outlined in FSSC 22000. Testing of environmental and raw product swabbing is done according to ISO 11290-1:2017 and ISO 11290-2:2017 relevant procedures and in an aseptic environment, as some of these findings may be included or make part of final data analysis.

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Chapter 2: Literature review of *L. monocytogenes* as a robust environmental pathogen associated with whole and RTE prepared-fruit

Listeriosis is a bacterial infection in humans caused primarily by the foodborne pathogen *Listeria monocytogenes* (Batt, 2014). *L. monocytogenes* has been recognised as a major foodborne pathogen since the 1980s (Abram *et al.*, 2012; Batt, 2014), although the organism was first described many years before this in the 1920s by EGD Murray (Murray *et al.*, 1926). *L. monocytogenes* has most commonly been associated with deli-meats, soft-ripened cheeses and fresh-cut vegetables (Orsi *et al.*, 2011). However, a number of recent outbreaks related to fruit (CDC, 2017) and the need for the industry to better manage *Listeria* species (spp.) at the point of production and processing, highlights the ongoing risk *Listeria* spp. and namely *L. monocytogenes* play in all food products. The prevalence of this genus of bacteria is well documented, and its robustness and environmental survivability makes it particularly difficult to eliminate from the food supply chain (Abram *et al.*, 2012; Carpentier, 2014; Orsi and Wiedmann, 2016).

Although *L. monocytogenes* and *L. ivanovii* are of greatest concern for consumer food safety, monitoring of all *Listeria sensu strictu* spp. (*L. monocytogenes*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. innocua* and *L. marthii*) is a practice employed by industry to better track and predict *L. monocytogenes* within their processing facility (Orsi and Wiedmann, 2016). It is for this reason that literature concerning *Listeria sensu strictu* spp. within the environment will be reviewed along with literature specific to *L. monocytogenes*. This pathogen and its relationship with fresh-cut fruit will be compared and discussed while the role of ready-to-eat (RTE) foods and consumer food safety and risk communication will be highlighted to contextualise the risk this organism plays in the food industry.

2.1 *Listeria*

The genus *Listeria* is widely distributed in the environment and is made up of over 20 species (Leclercq *et al.*, 2019; Nwaiwu, 2020). All the organisms within the genus *Listeria* are described as small rod-shaped Gram-positive bacteria (Adams and Moss, 2008). Of the more

than 20 species, two have been identified as pathogens: *Listeria monocytogenes* and *Listeria ivanovii* (Freitag *et al.*, 2009; Guillet *et al.*, 2010). *L. monocytogenes* is considered an important foodborne pathogen responsible for the illness listeriosis in humans (Orsi and Wiedmann, 2016). *L. ivanovii* can cause listeriosis in humans, however, this organism is generally associated with animals (Orsi and Weidmann, 2016). When considering the importance of mapping and detection of *Listeria* spp., it is not uncommon to use the presence of the non-pathogenic species to spot the conditions suitable for the proliferation and persistence of *L. monocytogenes* within the food supply chain and processing facilities (Orsi and Weidmann, 2016). *Listeria* as a genus can be separated into two main classifications, *Listeria sensu strictu* and *sensu lato*. The most prevalent species fall into the category *sensu strictu*, these include *L. monocytogenes*; *L. seeligeri*; *L. ivanovii*; *L. welshimeri*; *L. innocua* and *L. marthii*. The species within *sensu strictu* are well documented and widely distributed, often found in a range of different environments (Orsi and Weidmann, 2016). In two of the larger research studies on the presence of species from the genus within the natural environment, it was noted that *L. seeligeri* was the most dominant (Orsi and Weidmann, 2016). One study (Stea *et al.*, 2015) noted that *L. monocytogenes* was the dominant species isolated from both rural and urban water sources as well from urban environments (Orsi and Weidmann, 2016). *L. ivanovii* is not often detected in environmental sampling but has typically been isolated from farm environments (McAuley *et al.*, 2014), however, the proposed low detection of this species has been postulated as a result of selection bias during enrichment and enumeration caused by the tailoring of laboratory techniques for *L. monocytogenes* (Orsi and Weidmann, 2016). Other studies have found some association between species within the genus and certain environments (Linke *et al.*, 2014).

In general, there is no definite evidence to suggest that certain *sensu strictu* species are associated with any particular environment (Orsi and Weidmann, 2016), because of the nonconformity in environments throughout the food supply chain. Certain environmental factors have been identified as being associated with the presence of *Listeria* species. Linke *et al.* (2014) performed a study in Austria which determined that *Listeria* spp. were more likely to be isolated from soil samples with a low moisture content, neutral pH and an even mixture of both sand and humus. This study also determined lower numbers of *Listeria* spp. in samples from winter.

In order to effectively manage this genus and pathogen, several studies have been done to investigate the various means by which survival in the natural and industrial environment is

enabled, with specific focus on *L. monocytogenes* and the characteristics unique to each serotype that divide the species into four distinct genetic clades.

Classification of *L. monocytogenes* serotypes into four different phylogenetic lineages is based on single-nucleotide polymorphism (SNP) analysis of three chromogenic regions which include: *prfA, plcA, hly, mpl, actA, plcB, lmo0206; lmo0298, lmo0299, lmo0300,* and *inlA, inlB*. These phylogenetic lineages allow *L. monocytogenes* serotypes to be better characterised in their ability to survive certain environmental niches, and to elicit particular responses in patients (Ward *et al.*, 2008; Orsi *et al.*, 2011). The two most prevalent lineages are I and II, both of which account for the majority of listeriosis cases (Orsi *et al.*, 2011), whilst lineage III is more typically associated with animal cases of listeriosis and lineage IV is uncommon (Orsi *et al.*, 2011).

Lineage I isolates (1/2b, 3b, 3c, 4b) are more typically found in cases of human listeriosis. The higher prevalence in clinical cases is often attributed to the serotypes in this lineage having a full length *inlA*, and thus a greater propensity for internalin production, essential to virulence (Schmid *et al.*, 2005; Chen *et al.*, 2020).

Lineage II isolates (1/2a, 1/2c, 3a) have been shown to be more greatly associated with the environment in comparison to lineage I isolates. Isolates in lineage II typically have high recombination rates, plasmid retention and high genetic diversity, all of which may contribute to environmental robustness (Haase *et al.*, 2014).

2.1.1 *Listeria* species

Listeria sensu strictu genomes are similar throughout the species and have a number of shared genes (den Bakker *et al.*, 2010) further characterised by a high number of internalin genes (den Bakker *et al.*, 2010). *Listeria sensu strictu* is comprised of *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, *L. marthii* and *L. innocua*. All these organisms together form a conserved monophyletic group within the genus. *L. grayi*, is deemed as loosely related to those within *Listeria sensu strictu*.

L. grayi is classified as part of the *Listeria sensu lato*, although it shares recent common ancestors, with the *Listeria sensu strictu*, represented as the first and second black circle as seen in Figure 2.1, and has a closely related phylogeny. *L. grayi* is the oldest characterised organism within *Listeria sensu lato* (Orsi and Wiedmann, 2016) with some of the key

differentiating characteristics between *L. grayi* and other *Listeria sensu strictu* species deemed to be because of several events in the genetic history of the genus (Chiara *et al.*, 2015; Orsi and Wiedmann, 2016).

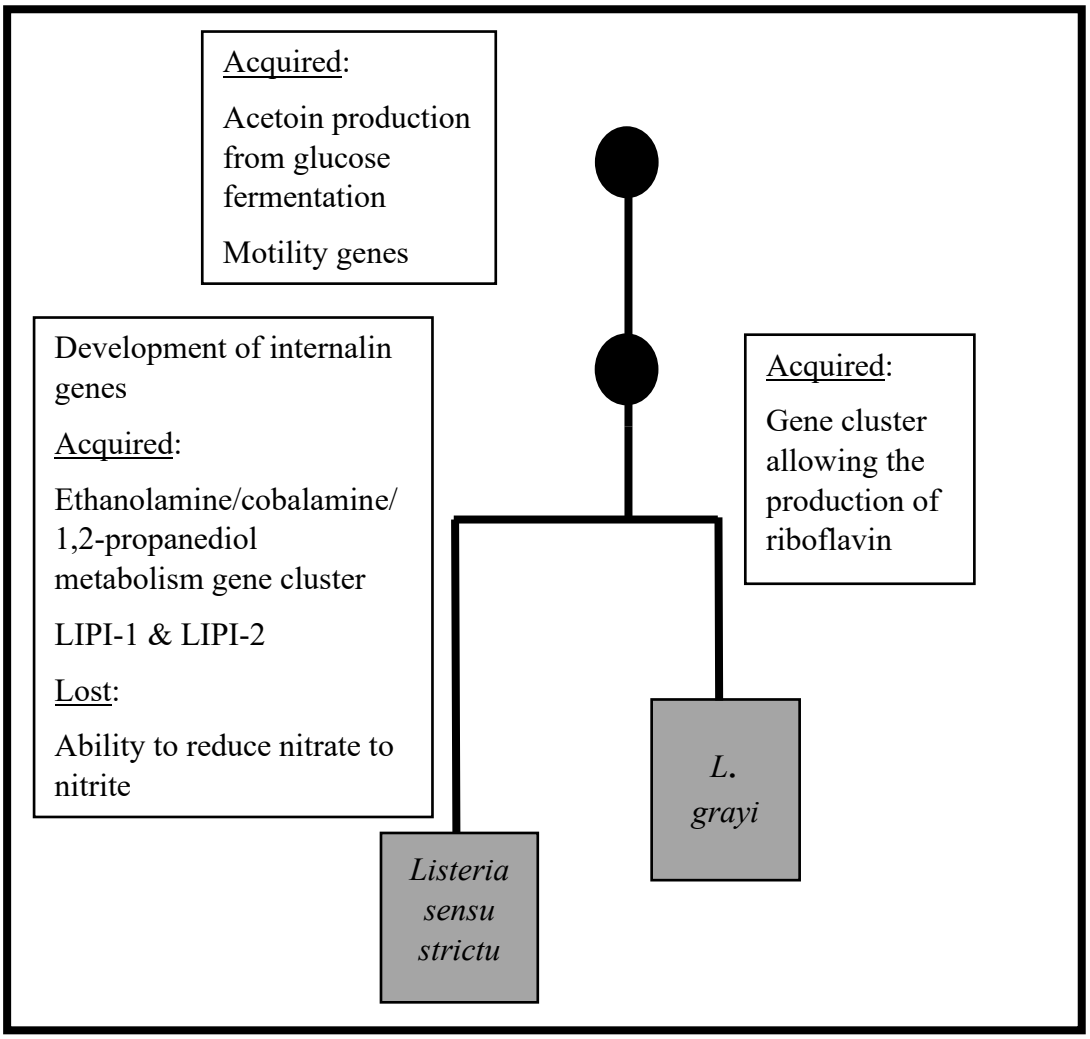

Figure 2.1 Diagram of genetic heritage of *L. monocytogenes* (Orsi and Wiedmann, 2016).

Listeria sensu strictu species have several phenotypic characteristics that are shared, as well as a number of phylogenetic differences based on historical evolutionary changes (Toledo-Arana *et al.*, 2009; Chiara *et al.*, 2015; Orsi and Wiedmann, 2016). Key attributes shared by all species within *Listeria sensu strictu* as well as their differences is illustrated in Table 2.1.

Table 2.1 Differences between *Listeria sensu strictu* species and *L. grayi*

***Listeria* spp.**	**Differentiating characteristics**	**Shared phenotypic characteristics**	**Metabolites that can be fermented**	**Metabolites that cannot be fermented**
L. monocytogenes	Phosphatidylinositol-specific phospholipase C (PI-PLC) Capable of haemolysis	Psychrotrophic nature allowing growth at 4^{O}C; motility; production of the enzyme catalase; inability to reduce nitrate to nitrite; and positive for Voges-Proskauer test – the inability to use the butanediol pathway in the fermentation of glucose to yield acetoin	D-arabitol, α-methyl D-glucoside, cellobiose, D-fructose, D-mannose, N-acetylglucosamine, maltose, and lactose	Inositol, L-arabinose, and D-mannitol
L. *ivanovii*	Phosphatidylinositol-specific phospholipase C (PI-PLC) Capable of haemolysis Fermentation of D-ribose and D-xylose			
L. innocua	Common – inability to cause haemolysis Uncommon – strain specific haemolytic capability Inability to ferment D-xylose Fermentation of glycerol			
L. *seeligeri*	Capable of haemolysis Fermentation of D-xylose but not D-ribose or D-tagatose			
L. welshimeri	Fermentation of D-tagatose and D-xylose			
L. marthii	Incapable of fermenting sucrose			
L. grayi	Fermentation of D-mannitol, D-ribose. Strains vary in their ability to reduce nitrate to nitrite		D-arabitol, α-methyl D-glucoside, cellobiose, D-fructose, D-mannose, N-acetylglucosamine, maltose, lactose and D-mannitol	Inositol, L-arabinose

(Toledo-Arana *et al.*, 2009; Chiara *et al.*, 2015; Orsi and Wiedmann, 2016)

The role that other species within the genus may play as potential reservoirs for genetic material to enhance the robustness and virulence of *L. monocytogenes*, and vice versa, is not well characterised (McLaughlin *et al.*, 2011; Zitz *et al.*, 2011; Gómez *et al.*, 2014; Moura *et al.*,

2019). The industrial food environment creates several situations enabling bacteria to interact and transfer genetic material (Katharios-Lanwermeyer *et al.*, 2012). One of the more robust *Listeria* spp. in the processing environment is non-pathogenic *L. innocua* (Barbalho *et al.*, 2005; Carvalheira *et al.*, 2010; Katharios-Lanwermeyer *et al.*, 2012; Vongkamjan *et al.*, 2015). It is important to characterize the resistance of these organisms – and other non-pathogenic *Listeria* – to the pressures in the food production environment. In fact, it has been found that non-pathogenic *Listeria* spp. are more likely than *L. monocytogenes* to have antibiotic resistance and hold plasmids, highlighting the potential role they may play in the genetic diversity of pathogenic *L. monocytogenes* (Antunes *et al.*, 2002; Davis and Jackson, 2009).

Phenotypic characteristics alone cannot be relied on for identification of a species, as atypical strains of species have been identified, including non-haemolytic *L. monocytogenes* as well as pathogenic *L. innocua*. The identification of a species characterised as non-pathogenic in the cause of bacteraemia and meningitis (Perrin *et al.*, 2003; Favaro *et al.*, 2014) indicates the need to better understand the genetic evolution of this genus and the potential for horizontal gene transfer between species within the genus.

2.1.1.1 Atypical haemolytic *L. innocua*

Non-pathogenic *Listeria* species have been implicated in fatal human infection. *L. seeligeri*, *L. welshimeri* and *L. innocua* have been identified in humans in the clinical setting (Rocourt *et al.*, 1986; Andre and Genicot, 1987; Perrin *et al.*, 2003; Favaro *et al.*, 2014). Although *L. seeligeri* is characterised as being capable of haemolysis it is deemed as being non-pathogenic, as is also the case with *L. innocua* (Perrin *et al.*, 2003; Favaro *et al.*, 2014; Moura *et al.*, 2019) with haemolytic *L. innocua* isolated from a range of products across multiple continents (Johnson *et al.*, 2004; Milillo *et al.*, 2012b; Moreno *et al.*, 2012). *L. innocua* is deemed to be the closest genetic relative of *L. monocytogenes* within *Listeria sensu strictu* (Doumith *et al.*, 2004; Johnson *et al.*, 2004). This means better understanding of this species can allow insight into the potential for genetic transfer and the genomic ancestry of *L. monocytogenes*.

L. innocua is one of the most abundant *Listeria* spp. in the industrial setting (Moreno *et al.*, 2012; Orsi and Wiedmann, 2016); however, the occurrence of deadly infections linked to this organism raise concern regarding the potential risk associated with the transfer of virulence related determinants (Moreno *et al.*, 2012; Favaro *et al.*, 2014; Orsi and Wiedmann, 2016). Evidence identifying *L. innocua* as the cause in fatal bacteraemia as well as meningitis in

patients over the age of 60 has been reported by Perrin *et al.* (2003) and Favaro *et al.* (2014), respectively. These isolates as well as other *L. innocua* found within the industrial environment allow for the genetic characterisation of these virulent atypical strains, allowing insight into the genetic relatedness of *L. innocua* and *L. monocytogenes* (Johnson *et al.*, 2004; Chiara *et al.*, 2015; Orsi and Wiedmann, 2016).

Favaro *et al.* (2014) makes clear reference to the many similarities between *L. innocua* and *L. monocytogenes* and highlights the challenges in differentiating these organisms through standard biological and serological protocols. *L. monocytogenes* can be detected through the identification of genes specific to the *Listeria* Pathogenicity Island 1 (LIPI-1) as well as internalins. One of the most common is the gene *hly* coding for haemolysin, this is also tested for phenotypically through the Christie, Atkins, Munch-Petersen (CAMP) test. The test is used for the identification of *Streptococcus agalactiae* but can be used for the identification of haemolytic ability in *Listeria* spp. in the presence of L-haemolysin producing *Staphylococcus aureus* and *Rhodococcus equi.* Test streaks of *Listeria* spp. are in one direction at right angles to – without touching – the *S. aureus* and the *R. equi* streaks on a sheep blood agar plate. Typically haemolysis is increased in the area of the streak closest to *R. equi* (Allerberger, 2003).

In the case of bacteraemia caused from *L. innocua*, serovar 6a was identified (Perrin *et al.*, 2003). Serovar 6a has been the subject of several studies and is often characterised as atypically haemolytic (Perrin *et al.*, 2003; Moreno *et al.*, 2012; Moura *et al.*, 2019) and may represent the most retro-genetic ancestor of *L. monocytogenes* in the *L. innocua* species. The isolate in the case of fatal bacteraemia showed no evidence of beta-haemolysis – consistent with *L. innocua* – but an uncharacteristic positive result was observed when the CAMP test was performed (Perrin *et al.*, 2003; Johnson *et al.*, 2004). In contrast, the *L. innocua* isolate responsible for bacterial meningitis produced a negative result when undergoing the CAMP test (Favaro *et al.*, 2014). Results of the CAMP test should be scrutinised, as interpretation of haemolytic reactions can be inconsistent (Vázquez-Boland *et al.*, 1992; McLauchlin, 1997; Allerberger, 2003).

Another interesting comparative between the case of bacteraemia and meningitis is described by Perrin *et al.* (2003) with no evidence of compromised immunity. In the case described by Favaro *et al.* (2014), however, the patient was deemed as having an increased risk due to the treatment of rheumatoid arthritis with etanercept and corticosteroids – both of which have been

linked to increased susceptibility to infection by *Listeria* and may have been an aggravating factor (Rivero *et al.*, 2003; Schett *et al.*, 2005; McCaffrey *et al.*, 2012; Mathews *et al.*, 2014).

Johnson *et al.* (2004) also investigated atypical *L. innocua* isolates capable of β-haemolysis and incapable of fermenting L-rhamnose. Their evidence concluded that LIPI-1 and associated genes (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, and *actA*) were present. Further investigation elucidated that the LIPI-1 regions in the atypical *L. innocua* were unlike those in *L. monocytogenes* and were phylogenetically different, however genetic analysis revealed possible ancestral links between *L. monocytogenes* serogroup 4a and *L. innocua* serovar 6a (Volokhov *et al.*, 2007). Moreno *et al.* (2012) observed high genetic similarity between *L. innocua* serovar 6a and *L. monocytogenes* serovar 4b. The hypothesis that *L. innocua* and *L. monocytogenes* serogroup 4 are closely related has been expressed by Doumith *et al.* (2004), and is further supported by the findings of Volokhov *et al.* (2007) and Moreno *et al.* (2012).

Two isolates from the study by Johnson *et al.* (2004) were subject to further investigation by Volokhov *et al.*, (2007) who confirmed that LIPI-1 was present, along with the virulence gene *inlA*, related specifically to *L. monocytogenes*. Also present was a genetic region not dissimilar to that of *inlB*. This was deemed a modified *inlAB* operon that lacked an *inlB* homologue (Volokhov *et al.*, 2007; den Bakker *et al.*, 2010). In contrast, the presence of virulence genes *inlA* and *inlB* were reported in the isolate responsible for meningitis (Favaro *et al.*, 2014), the presence of *inlB* was also observed by Moreno *et al.* (2012) in atypical *L. innocua* isolated from a pork production environment. This is something which has not been noted in other atypical haemolytic *L. innocua* (Johnson *et al.*, 2004; Volokhov *et al.*, 2007; Moreno *et al.*, 2012; Moura *et al.*, 2019). *InlA* and *inlB* are part of the *inlAB* operon and are responsible for the production of surface internalins fundamental to causing infection through the invasion of several types of non-phagocytic cells (Lingnau *et al.*, 1995; Hamon *et al.*, 2006).

In terms of the diversity within haemolytic *L. innocua*, Moura *et al.* (2019) observed two distinct genetic clades of atypical haemolytic *L. innocua* both containing LIPI-1. In the two clades of *L. innocua* it was noted that Finnish isolates CLIP 2016/00427 and CLIP 2016/00428 belonged to serotype 6a and formed a monophyletic group, whereas FSL J1-023 belonged to serotype 4ab and the genetic clade within which it fell showed a truncated *mpl* gene (Moura *et al.*, 2019). This finding was supported in part by Volokhov *et al.* (2007) who observed an intact *mpl* gene in the atypical *L. innocua* isolate PRL/NW 15B95 which belonged to serovar 6a. The truncation of *mpl* negatively affects the ability of the organism to escape vacuoles (Alvarez and

Agaisse, 2016) and may negatively affect the ability of the organism to adequately survive intracellular invasion. Conversely Favaro *et al.* (2014) were able to identify the gene *mpl* as well as *inlB* in the *L. innocua* isolate implicated in human meningitis, but were not able to identify *actA*, which has been identified in other atypical *L. innocua* isolates (Volokhov *et al.*, 2007). It should also be noted that all atypical *L. innocua* have not showed evidence of the *inlC* gene (Perrin *et al.*, 2003; Favaro *et al.*, 2014; Moura *et al.*, 2019), besides in the isolate PRL/NW 15B95 analysed by Volokhov *et al.* (2007). This may also have been a contributing factor in atypical *L. innocua* being less virulent than *L. monocytogenes* (Moura *et al.*, 2019) as the protein internalin C was shown to play a crucial role in *in vivo* cell-to-cell spread in mice (Leung *et al.*, 2013).

Differences like this are seen throughout the *Listeria* genus as evidence of their transition from facultative pathogen to obligate saprotroph, which involved the loss of genes and decrease in genome size (Moran, 2002; den Bakker *et al.*, 2010). It is largely hypothesised that the ancestor of *Listeria* possessed all virulence related genes which were subsequently lost in varying degrees, allowing the emergence of new species. In the paper by den Bakker *et al.* (2010), a description of the evolution of *Listeria sensu strictu* towards saprotroph is described as having taken place four times, coinciding with speciation of the genus. These are described as outlined by den Bakker *et al.* (2010) in Table 2.2 below:

Table 2.2 Evolutionary events linked to the formation of different species within *Listeria sensu strictu* (den Bakker *et al.*, 2010)

Event	Description	Species produced from event	Secondary event	Species produced from event
1	Loss of *inlAB* operon and *inlC*. Retention of *prfA* cluster	*L. seeligeri*	Loss of *prfA* cluster	Non-haemolytic *L. seeligeri*
2	Loss of *inlAB* operon, *inlC* and *prfA* cluster	*L. welshimeri*	-	-
3	Loss of *inlB* and *inlC*	*L. innocua*	Loss of *prfA* cluster and *inlA*	Non-haemolytic *L. innocua*
4	Loss of *inlAB* operon, *inlC*, and *prfA* cluster	*L. marthii*	-	-

Atypical haemolytic *L. innocua* has two hypothesised origins. One hypothesis is that the virulence gene cluster was acquired through a form of transformation or phage-mediated transfer (den Bakker *et al.*, 2010). This was suggested after the identification of genes involved in the uptake of DNA in the genomes of both *L. monocytogenes* and *L. innocua* (Schoenfeld, 1976). The partial transfer of LIPI-1 but no other virulence determinants through horizontal gene transfer may explain the avirulence observed in the haemolytic *L. innocua* isolates (Johnson *et al.*, 2004). These atypical *L. innocua* isolates may also represent a stage in the evolution of *L. innocua* from a common ancestor with *L. monocytogenes* (Volokhov *et al.*, 2007; Moreno *et al.*, 2012). The risk posed by the occurrence of atypical haemolytic *L. innocua* is further supported by the unusual occurrence of atypical *L. innocua* which possess LIPI-3, a pathogenicity island associated exclusively with lineage I isolates of *L. monocytogenes* (Clayton *et al.*, 2014). The presence and functionality of LIPI-3 was varied throughout atypical *L. innocua* isolates, representing the stages in reductive evolution. Nonetheless, the retention of the LIPI-3 cluster by a large proportion of strains is unexpected (Clayton *et al.*, 2014).

The findings of Johnson *et al.* (2004); Volokhov *et al.* (2007) and Moura *et al.* (2019) suggest that these atypical haemolytic isolates of *L. innocua* represent an intermediary evolutionary stage, and that the presence of LIPI-1 and *inlA* and the absence of *inlB* make the potential for horizontal gene transfer from pathogenic *L. monocytogenes* unlikely (Schmid *et al.*, 2005; Maury *et al.*, 2017). *L. innocua* may be identified as pathogenic *L. monocytogenes* because of the detection of *hly* and other virulence genes located in LIPI-1 (Orsi and Wiedmann, 2016), this is something that should be considered when selecting marker genes for species identification. Furthermore, these atypical strains may serve as reservoirs for virulence genes potentially transferable to other species in the genus (Volokhov *et al.*, 2007; Moreno *et al.*, 2012).

2.2 *L. monocytogenes* survival in the natural and industrial food environment

The ability of *L. monocytogenes* to colonise and persist in several challenging environments is the subject of many research studies, each one revealing a more adaptable and robust organism than previously thought. *Listeria* spp. can be isolated from soil, decaying vegetation, water and within several animals including humans (Adams and Moss, 2008; Abram *et al.*, 2012; Orsi and Wiedmann, 2016). The means by which *Listeria* spp. can proliferate in the natural environment is often attributed to the genome of the species which possesses a large number of genes that facilitate survival (Hauf *et al.*, 2019). Two examples of this is the more than 30 phosphotransferase systems, in reference strain EGD-e, used for the uptake of different sugars (Barabote and Saier, 2005) as well as the range of cellular functions regarding nutrient uptake and antibiotic resistance attributed to the adenosine triphosphate (ATP)-binding cassette (ABC) transporters coded for in the *Listerial* genome (Fraser *et al.*, 2000; Gopal *et al.*, 2010; Liu *et al.*, 2013b). The drug-resistant capabilities of this organism are still being understood through recent findings (Hauf *et al.*, 2019) indicating antibiotic resistance to aurantimycin A, an antibiotic produced by *Streptomyces aurantiacus* (Gräfe *et al.*, 1995). As both *Streptomyces* and *Listeria* spp. are associated with soil (Orsi *et al.*, 2011; Hauf *et al.*, 2019) and the genes responsible for this resistance – *lieAB* – are expressed in the environment and not likely within the human host (Hauf *et al.*, 2019), it can be seen that *L. monocytogenes* has adapted survival mechanisms specifically for the natural environment. Other studies also discuss the potential that protozoa like *Acanthamoeba* may play in facilitating transmission of the organism through

internalisation, however, this hypothesis was not fully accepted and recent findings have determined this was not the case as the predatory protozoa do not act as a reservoir – *L. monocytogenes* was unable to survive within *Acanthamoeba* (Abram *et al.*, 2012; Doyscher *et al.*, 2013). Having said this, evidence that *L. monocytogenes* was immobilised and formed aggregates on the exterior of the *Acanthamoeba* cell membrane – termed backpacks (Doyscher *et al.*, 2013) – were not found to be specific to *L. monocytogenes* and instead their formation was found to be a feeding strategy of the *Acanthamoeba* (Doyscher *et al.*, 2013).

Soil is the most commonly associated natural reservoir for *Listeria* spp., however, the presence of the organism on both vegetation and in water exemplify its ubiquity (Fenlon, 1999; Ivanek *et al.*, 2007; Hellberg and Chu, 2016; Hauf *et al.*, 2019). The saprophytic nature of *Listeria* spp. means that their presence in the soil may be as a result of contamination from other natural sources such as decaying vegetation, animal faeces and sewage (Ivanek *et al.*, 2007). By better understanding the soil and how *Listeria* spp. proliferate, a more effective method of management can be implemented to prevent cross-contamination or transfer of the organism to new environmental niches. One thing to note is that the presence of *Listeria* spp. is higher at the soil surface than deeper down and is generally in a greater abundance where animals are present (Ivanek *et al.*, 2007). McLaughlin *et al.* (2011), highlight the ability of *L. monocytogenes* to survive for extended periods in soil under laboratory conditions, with multiplication even observed under winter conditions in sterile soil (Botzler *et al.*, 1974). Although there is evidence of their survival in soil, the initial numbers of *Listeria* spp. present declines with time and the length that they can survive has a strong link to the soil pH, with lower pH being unfavourable (McLaughlin *et al.*, 2011). One major association with survival in soil was found to be the motility of the organism, as deletion mutants that lacked motility demonstrated reduced survival (McLaughlin *et al.*, 2011). This is an important finding when analysing the presence of the organism in the processing environment as motility is linked to biofilm formation, a survival strategy enabling *Listeria* spp. to persist in the environment, specifically when a surface is in contact with a liquid medium (Mayansky *et al.*, 2012; Carpentier, 2014; Piercey *et al.*, 2016; Vázquez-Sánchez *et al.*, 2017). *L. monocytogenes* was also seen to show increased survivability at lower temperatures; this was more than likely as a result of the increased capacity to survive stress when the metabolic rate is lowered (McLaughlin *et al.*, 2011). Higher temperatures also catalysed moisture loss in the soil, however, this was determined to only affect the survivability of the organism over prolonged periods (McLaughlin *et al.*, 2011). The amount of time which *Listeria* spp. can survive in soil

was also reduced by the introduction of aerobic microbiota – an indication of the adverse effect that the limiting of nutrients can play over time (McLaughlin *et al.*, 2011). The role the natural environment plays in the survival and growth of *Listeria* spp. is of substantial importance when considering its transmission to the processed environment. This is especially true of soil, which in many cases is contaminated by water and faeces (Ivanek *et al.*, 2007).

Within the production environment *Listeria* spp. and most importantly *L. monocytogenes* exhibit high stress tolerance and survivability which allows for their persistence and proliferation. The processing environment already selects for *Listeria* spp. by virtue of the environmental conditions which are designed specifically for the inhibition of the majority of pathogens and spoilage microorganisms through microbial hurdles like low operating temperatures; lowered pH; and inhibitory salt concentrations, all of which *Listeria* spp. (namely *L. monocytogenes*) show increased tolerance against (Forsythe, 2007; Lourenço *et al.*, 2011). This also limits the number of microbiotas within the facility and thus removes a great deal of potential for any competitive inhibition of the organism (Heir *et al.*, 2018). Eradicating those microorganisms capable of competing with pathogens there is the inadvertent selection for the growth of these pathogens (Bowen *et al.*, 2006).

2.2.1 Impact of climate and weather changes

The role of weather and seasonal changes can affect the dissemination and survivability of pathogens within the agricultural environment and food supply chain, especially in the case of a saprophytic soil borne organism like *L. monocytogenes* (Ivanek *et al.*, 2007).

Air temperature, water temperature and rainfall patterns are some of the prevalent factors affecting the occurrence of foodborne illnesses, with seasonal changes being linked to contamination and foodborne illness (Smith and Fazil, 2019). Soil splash as a result of rainfall has been identified as a potential route for the transfer of *Listeria* onto produce (Girardin *et al.*, 2005), with greater droplet sizes associated with thunderstorms contributing to greater soil splash, whilst greater dispersal of *Listeria* from short periods of rainfall has also been noted (Hellberg and Chu, 2016).

Findings by McLaughlin *et al.* (2011) indicate that moisture content is only a determining factor in the decline of *Listeria* in soil over prolonged periods, however, higher levels of soil contamination during harvesting has been seen in wet conditions (Monaghan and Hutchison,

2012). Importantly, high cumulative rainfall has been shown to reduce the presence of *L. monocytogenes* (Wilkes *et al.*, 2009), thought to be as a result of higher *L. monocytogenes* in run-off water (Miettinen and Wirtanen, 2006). The role of watercourses in the dispersal of pathogens is also an important aspect of seasonal and climatic changes as flooding of rivers has shown to contribute to greater *L. monocytogenes* prevalence in soil (Linke *et al.*, 2014).

Dry conditions with sunlight and elevated temperatures have shown to decrease microbial loads in soil surfaces, in contrast to the persistence of bacteria noted in cooler, wetter conditions with lower levels of sunlight (McLaughlin *et al.*, 2011; Monaghan and Hutchison, 2012), this may be attributed to lower metabolic rates, increased moisture retention or a competitive advantage over other microbiota in the soil (McLaughlin *et al.*, 2011; Hellberg and Chu, 2016; Vivant *et al.*, 2017; Smith *et al.*, 2018). However, incidences of listeriosis are more common during fluctuations in ambient temperature and high summer temperature peaks due to the negative impact this has on maintaining the cold-chain and the change in replication patterns of *L. monocytogenes* in contaminated products (Chersich *et al.*, 2018). Furthermore, prolonged dry seasons contribute to *Listeria* contamination in more indirect ways, as water scarcity may hamper hand washing, cleaning and sanitisation as well as increased use of surface water for irrigation which is more likely contaminated with *Listeria* than potable water (Chersich *et al.*, 2018).

Changes in climate and seasonal effects should be taken into consideration when developing control strategies for *L. monocytogenes.* Although numerous factors may influence *L. monocytogene's* ability to survive in the natural environment, the role of temperature, sunlight and rainfall patterns can be monitored to reduce the levels of contamination of fresh and whole produce before it enters the food-processing environment where it may persist and compromise food safety.

2.2.2 Biofilms

One of the major means by which *Listeria* spp. can persist in the processing environment is through the formation of biofilms (Schlech *et al.*, 1983). Biofilms represent the sessile state of the microorganism and involve the adhesion and colonisation of a surface (Di Ciccio *et al.*, 2012; Carpentier, 2014), thus allowing persistence through increased resistance to desiccation, ultraviolet (UV) light, as well as sanitisers and biocides (Di Bonaventura *et al.*, 2008). Biofilms typically form in niches or reservoirs within the production facility enabling their proliferation.

These are typically hard-to-clean areas like crevices or areas which do not drain well (Carpentier, 2014; Stea *et al.*, 2015). This is an important consideration as badly-designed equipment which cannot be adequately cleaned creates the opportunity for the persistence of *Listeria* spp. within biofilms (Carpentier, 2014).

Within their niche, microorganisms are involved in a range of interactions, some symbiotic and others competitive. These complex interactions have been demonstrated in biofilms with the bacterium *L. monocytogenes* (Habimana *et al.*, 2011; Elias and Banin, 2012). Some of the fundamental drivers for the conversion of the organism from the planktonic state to the sessile state are the colonisation of a favourable environment, the benefits of communal existence which include genetic transfer and intercell signalling and protection from the bulk phase of the environment which contains harmful compounds (Jefferson, 2004). An example of the role the microbial community in biofilms plays in enhancing the survivability of *L. monocytogenes* is the observed reduction in susceptibility to sanitisers when grown in a co-culture biofilm with *Pseudomonas aeruginosa* (Lourenço *et al.*, 2011). Other observations, Piercey *et al.* (2016), showed decreased lethality of sanitisers at increased biofilm maturity, an indicator of how persistent strains within biofilms can become increasingly robust. The biofilm itself, even when the microorganism has no antimicrobial resistance, serves to protect and enable the survival of many of the organisms within the community as a result of limited biocide diffusion within the biofilm; neutralisation of the biocide by the biofilm matrix; presence of tolerant dormant cells; and different regulation of gene expression (Costerton *et al.*, 1995; Poole, 2008; Van Acker *et al.*, 2014; Møretrø *et al.*, 2017).

L. monocytogenes itself does not have a great propensity to form monoculture biofilms in the processing environment (Nilsson *et al.*, 2011), however, its adherence and biofilm-forming capabilities may be supported through colonisation of food-contact surfaces (FCS) by other bacteria (Habimana *et al.*, 2009 as cited by Liu *et al.*, 2016).

It cannot be denied that biofilms in essence are environments that are by their nature symbiotic, however, Heir *et al.* (2018) demonstrated the competitive effect that *L. innocua* and gram-negative bacteria have on the growth of *L. monocytogenes* in multiple bacteria biofilms. It was found that *L. monocytogenes* strains show different capabilities to grow and compete in biofilms with multiple different bacteria. The different strains of *L. monocytogenes* were categorised as either being strong or weak competitors. Growth inhibition by *L. innocua* was observed, with the inhibition exacerbated with the inclusion of Gram-negative bacteria commonly associated with *L. monocytogenes* biofilms in food-processing environments. All

strains in biofilms with mixed Gram-negative bacteria showed no differences in the level of growth inhibition (Heir *et al.*, 2018). In biofilms with *L. innocua* there was evidence of individual *L. monocytogenes* strains which could withstand inhibition while others could not. The strains which were not strong competitors in biofilms with *L. innocua* were further inhibited in multispecies biofilms that contained both *L. innocua* and mixed Gram-negative bacteria (Heir *et al.*, 2018). These findings suggest that strong competitor strains of *L. monocytogenes* will benefit from this selective advantage and will exhibit improved growth and persistence (Heir *et al.*, 2018). Inhibition of *L. monocytogenes* in mixed-culture biofilms is thought to be as a result of both direct and indirect mechanisms like the competition for nutrients, the production of antimicrobials and low-temperature growth capabilities (Heir *et al.*, 2018). All of these mechanisms are recognised by a number of research papers as playing a significant role in the inhibition of *L. monocytogenes* in the processing environment (Zilelidou *et al.*, 2016; Fagerlund *et al.*, 2017).

2.2.3 Sanitisers and biocides

L. monocytogenes resistance to quaternary ammonium compound (QAC) sanitisers like benzalkonium chloride (BC) is a growing concern in the food industry and is another one of the major means by which the organism is surviving in the modern supply chain (Allen *et al.*, 2016). QACs are an active ingredient in several sanitisers and the increase in antimicrobial resistance is largely thought to be as a result of their misuse, in which sub-lethal amounts are used (Buffet-Bataillon *et al.*, 2012; Allen *et al.*, 2016). Long-term exposure to QACs at sub-inhibitory concentrations selects for organisms with either acquired or intrinsic resistance to higher concentrations (McBain *et al.*, 2002; Poole, 2008; Smith *et al.*, 2008). QAC activity is based primarily on the N-alkyl chain which presents lipophilicity, allowing interaction with the acidic phospholipids within the bacterial membranes (Gilbert and Moore, 2005). These compounds solubilise hydrophobic cell membrane components and may disrupt and denature structural proteins and enzymes as part of their action (Gilbert and Moore, 2005). Bacterial resistance to these compounds is enabled through efflux pumps; reduced expression of porins; and mobile genetic elements that confer resistance (Buffet-Bataillon *et al.*, 2012; Allen *et al.*, 2016).

The greatest concern related to newfound antimicrobial resistance in *L. monocytogenes* is the impact it has on virulence and pathogenicity. Although there is a need for greater understanding

regarding this topic, research indicates a link between stress response and pathogenic process (Oliver *et al.*, 2009; Toledo-Arana *et al.*, 2009) most notably with the general response regu*lato*r σ^B which also regulates a number of virulence factors and therefore antimicrobials affecting σ^B activity influence stress response and invasion processes for host infection (Oliver *et al.*, 2009). This evidence precautions the use of clinical antimicrobials in food production as prior exposure to these agents may influence the regulation of certain genes and the physiology of the organism before human consumption (Allen *et al.*, 2016).

2.3 *Listeria* association with prepared-fruit

Although a number of high-profile listeriosis outbreaks have occurred as a result of deli meats and soft cheeses, fresh produce can be deemed a significant contributor to foodborne listeriosis (Martinez *et al.*, 2016). Fresh fruit and vegetables are promoted as part of a healthy lifestyle and nutritious diet, hence a *per capita* increase in consumption. This is noted with the increase in consumption of fruit like cantaloupe between 1980 and 2002 of 5.5 lb *per capita* in the US (Bowen *et al.*, 2006)

There are several concerns regarding the safety of fresh cut-produce, because of the lack of microbial elimination possible for these products. This is due to the negative impacts on quality and consumer acceptability, hence the need to control microbial safety through the appropriate use of cleaning and sanitiser application. *L. monocytogenes* is of concern for processed low-acid fruits like papaya, melon and watermelon (Penteado and Leitão, 2004). The potential for a listeriosis outbreak as a result of prepared-fruit products is steadily on the rise (Spadafora *et al.*, 2016). The importance that consumption of a variety of fresh and minimally processed fruits has on human health has been increasingly stressed, and has impacted on the popularity of these products amongst consumers (Niemira and Fan, 2012), specifically in South Africa, where RTE food products and health consciousness are growing dimensions of consumer needs (Ronquest-Ross *et al.*, 2015)

Due to the minimal processing employed for prepared-fruit and vegetables, there is a reliance on refrigeration temperatures to control and mitigate the potential growth and proliferation of pathogenic bacteria, however, *L. monocytogenes* is a highly capable psychrophile and can grow at temperatures of 0^OC and survive freezing. As a facultative anaerobe it is also capable of survival at low oxygen levels (Adams and Moss, 2008; Niemira and Fan, 2012). This alone raises a number of concerns with regards to the best practices required to control this organism

and ensure it does not contaminate a food product to the point that the consumer may contract listeriosis (Penteado and Leitão, 2004; Spadafora *et al.*, 2016). This control of temperature is not an effective means of controlling pathogens because of the many microenvironments that may have elevated temperatures and humidity typically associated with these factories (Liu *et al.*, 2013a). Furthermore, it was found that fresh-cut processing plants were home to a number of microorganisms with strong biofilm forming abilities; by extension, these bacteria increase the likelihood of pathogenic bacteria due to the opportunity to reside in a sessile state and develop within an ecological niche (Liu *et al.*, 2013a).

New measures employed for the control of *L. monocytogenes* on fruit products include the use of bacteriophages, which have shown to be effective in reducing the pathogens load on fresh-cut products like melons and pears (Oliveira *et al.*, 2014). The same has been noted with regards to the application of ultraviolet-C, shown to reduce *L. monocytogenes* on both cantaloupe and strawberry (Adhikari *et al.*, 2015), although it should be noted that the rough surface of cantaloupe was deemed to have a mitigating effect on microbial reduction. This is supported by the findings of Marik *et al.* (2020), noting the role of rougher surface characteristics in their ability to increase the likelihood of contamination, even in the case of avocado. Another aspect of *L. monocytogenes* growth on fruit surfaces is nutrient availability, and while the pulp of fruit is often an adequate environment for the growth of *L. monocytogenes*, limited nutrients on fruit surfaces negatively affect *L. monocytogenes* survivability; furthermore, high levels of natural microbiota provide a competitive environment which is also disadvantageous to the proliferation of *L. monocytogenes* (Marik *et al.*, 2020).

The occurrence of listeriosis outbreaks as a result of fruit has occurred in a limited number of cases, where stone fruit (Chen *et al.*, 2016a), melons (McCollum *et al.*, 2013) and caramel apples (Angelo *et al.*, 2017) have been implicated, all occurring in the USA.

2.3.1 RTE fruit products

RTE fruit products, although very seldom found to be vectors for pathogenic organisms (Willis *et al.*, 2016) have been shown to support the growth of *L. monocytogenes* as previously mentioned, with instances of certain combinations of fruit showing a greater propensity for growth than others (Ziegler *et al.*, 2018). Data regarding the prevalence of *L. monocytogenes* occurrence in RTE fruit products in the UK indicates contamination levels of concern, with studies reporting levels of contamination by *Listeria* between 5-8%, with significantly higher

levels in prepared melon products (Little and Mitchell, 2004; Willis *et al.*, 2016; Ziegler *et al.*, 2018). It was noted by Willis *et al.* (2016), that contamination by *L. monocytogenes* likely occurred at some point in the supply chain prior to sale at the retailer, either during preparation, packing or growing and is likely further aggravated by the range of conditions along the supply chain (Little and Mitchell, 2004).

One of the main aggravating factors in contamination of *L. monocytogenes* in prepared-fruit products lies in the growth advantages conferred by exposure of the fruit pulp, especially as a result of the relatively neutral pH of melons like cantaloupe and watermelon (6.2-6.7 and 5.8-6.0 respectively) (Willis *et al.*, 2016). The role of a more acidic pH in preventing the growth of *L. monocytogenes* is a significant factor (Ziegler *et al.*, 2018), as a result of *L. monocytogenes* inability to survive low pH (pH $\leq$ 4) (Milillo *et al.*, 2012a). Furthermore, the type of fruit showed no effect on *L. monocytogenes'* growth as much as low pH; this is important as different fruit have different pH values. However, pH values may differ for different fruit of the same type and as a result of ripeness, or from their inclusion in a product with other fruit (Ziegler *et al.*, 2018). The role of fruit mixtures can have a significant effect on the growth of *L. monocytogenes* compared to the individual components, as noted by Ziegler *et al.* (2018); this is in part due not only to altered pH values, but also the role of natural microbiota.

The importance of proper storage temperatures is vital in controlling *L. monocytogenes* levels during transport and storage at retail outlets. Although growth can even occur at 10^{o}C in fruit like papaya, watermelon and cantaloupe, this is far less than at higher temperatures of 20 and 30^{o}C (Penteado and Leitão, 2004). There is, however, contrasting evidence that temperature plays a less important role than pH and storage time (Ziegler *et al.*, 2018).

Exposure to the fruit pulp in the agricultural setting, through bruising, cracking or any form of external damage to the fruit surface, greatly enhances the potential for *L. monocytogenes* growth and colonisation especially when the fruit product is grown close to the ground (Willis *et al.*, 2006), in turn increasing the potential for contamination during slicing and prepping.

2.3.2 Cantaloupe

The greatest challenge to managing microbial contamination in the preparation of fruit is the processing steps of trimming, peeling, cutting and packaging, all of which are potential areas for the contamination of the product. The failure to detect contaminated food runs a major risk

of outbreaks and the number of listeriosis cases linked to fresh produce has grown in recent years, with the largest incidence in the USA in 2011 – where a multistate outbreak of listeriosis from cantaloupe resulted in the deaths of 32 and one miscarriage (McCollum *et al.*, 2013). This finding bears similarities to the majority of listeriosis outbreaks, especially of RTE products, where the post-processing environment or equipment has been the point of contamination (Bezanson *et al.*, 2018). A more recent outbreak occurred in 2018 in Australia with 20 cases, seven deaths and one miscarriage (Das, 2019).

The 2011 outbreak in the USA was linked to whole cantaloupe which had not previously been considered a vector for *L. monocytogenes.* There are several foodborne pathogens associated with melons, as they are grown in direct contact with the soil, creating potential avenues for contamination in the post-harvest and processing environment (Bowen *et al.*, 2006; Kwon *et al.*, 2018). Furthermore, there were several genetically-distinct strains of serotypes 1/2a and 1/2b, both of which had also been implicated in a febrile gastroenteritis outbreak in non-produce foodstuffs (Lomonaco *et al.*, 2013). One finding following the outbreak was the association of those particular strains to the packaging and processing environment, and not to the environment in which the fruit was grown (McCollum *et al.*, 2013). The potential for cross contamination from the surface of the fruit during cutting is a risk, however there is also significant risk of internalisation of the pathogen during processing post-harvest (Chen *et al.*, 2016b). Research investigating the listeriosis outbreak in Australia in 2018, linked to serotype 4b ST240 on contaminated rockmelon, are limited (Das, 2019). There are, however, some postulated causes for the outbreak, namely increased rainfall, dust storms and an unclean packing environment (Das, 2019)

As previously stated, *L. monocytogenes* is a persistent coloniser of soil and surface water (Orsi and Wiedmann, 2016). With the sharp rise in listeriosis outbreaks linked to fresh produce (CDC, 2017), the challenges to ensure food safety are more critical than ever. Cantaloupes are a crop of interest with regards to the prevalence of *Listeria* spp. as these melons are grown on the ground and are susceptible to contamination by not only the soil, but also irrigation water (Bezanson *et al.*, 2018). Of the 34 melon-associated outbreaks between 1973 and 2011, 19 (56%) were as a result of cantaloupe, followed by watermelon as the next most prevalent (Walsh *et al.*, 2015).

Cantaloupe as well as other melons provide adequate nutrients for *Listeria* spp. to survive and proliferate (Huang *et al.*, 2019), due to the relatively neutral pH of the fruit pulp, as well as the abundance of nutrients and sugars (Willis *et al.*, 2016; Ziegler *et al.*, 2018). *L. monocytogenes*

had a shorter lag phase on freshly-prepared and juiced melon – cantaloupe having the greatest lag phase, followed by honeydew and watermelon – than that of pineapple or radish (Huang *et al.*, 2019). Huang *et al.* (2019) argued that maintaining adequate refrigeration temperatures is critical to ensuring the safety and quality of produce and validate this in their research findings investigating the impact that temperature abuse plays in the proliferation of pathogens on melon, where *L. monocytogenes* was capable of slow but consistent growth at 4°C. This raised concerns regarding the safety of prolonged storage at refrigeration temperatures. Temperature abuse was shown to increase in growth of *L. monocytogenes*, attributed to the suitable composition of cantaloupe and other melons for this pathogen (Huang *et al.*, 2019). This is supported by previous work by Huang and Luo, (2015) who found that temperature abuse of freshly-cut cantaloupe allowed for significant growth of *L. monocytogenes.* Nyarko *et al.* (2016) support this, finding 1-2 log CFU/cm^2 increase in *L. monocytogenes* on stem sites and in rind juice at refrigeration temperatures. Nyarko *et al.* (2016) also found that *L. monocytogenes* numbers declined on the surface of cantaloupe regardless of temperature; this is in contrast to Martinez *et al.* (2016), who noted significantly higher ($p \geq 0,05$) growth on cantaloupe surfaces at both 8 and 25°C over seven days as opposed to 4°C for 21 days. The above conclusions provide substantial evidence to explain the findings of Bowen *et al.* (2006) and Walsh *et al.* (2015), who found 61% of outbreaks were as a result of prepared melon between 1984-2002, and that pre-cutting of melons was attributed to 77% of outbreaks between 1973-2011 respectively. These findings highlight the enhanced growth capability of *L. monocytogenes* in cantaloupe pulp.

The netted rind of cantaloupes provides several challenges with regards to microbial reduction of the raw material in the processing environment, namely because of the ease with which bacterial pathogens can adhere to this surface (Ukuku and Fett, 2002). The potential for cross contamination is highly probable during preparation, as cutting of the fruit may transfer pathogens from the rind to the edible pulp (Ukuku and Fett, 2002). One study by Wang *et al.* (2009) investigated the effect surface roughness has on bacterial adhesion and retention by comparing fruits of varying roughness and the retention and removal of *E. coli* O157:H7. Cantaloupe was classified as having a high surface roughness in comparison with avocado, pear and apple respectively. It was shown that an increase in the roughness of a surface, both metal and fruit, enables microbes on the fruit surface to remain protected and as a result causes less effective washing (Wang *et al.*, 2009). This is seen in Figure 2.2, where fruit with rougher surfaces are more likely to be contaminated with *L. monocytogenes*. Similar findings were

observed by Fransisca and Feng, (2012) who found that surface roughness had a negative relationship with the removal of microbes.

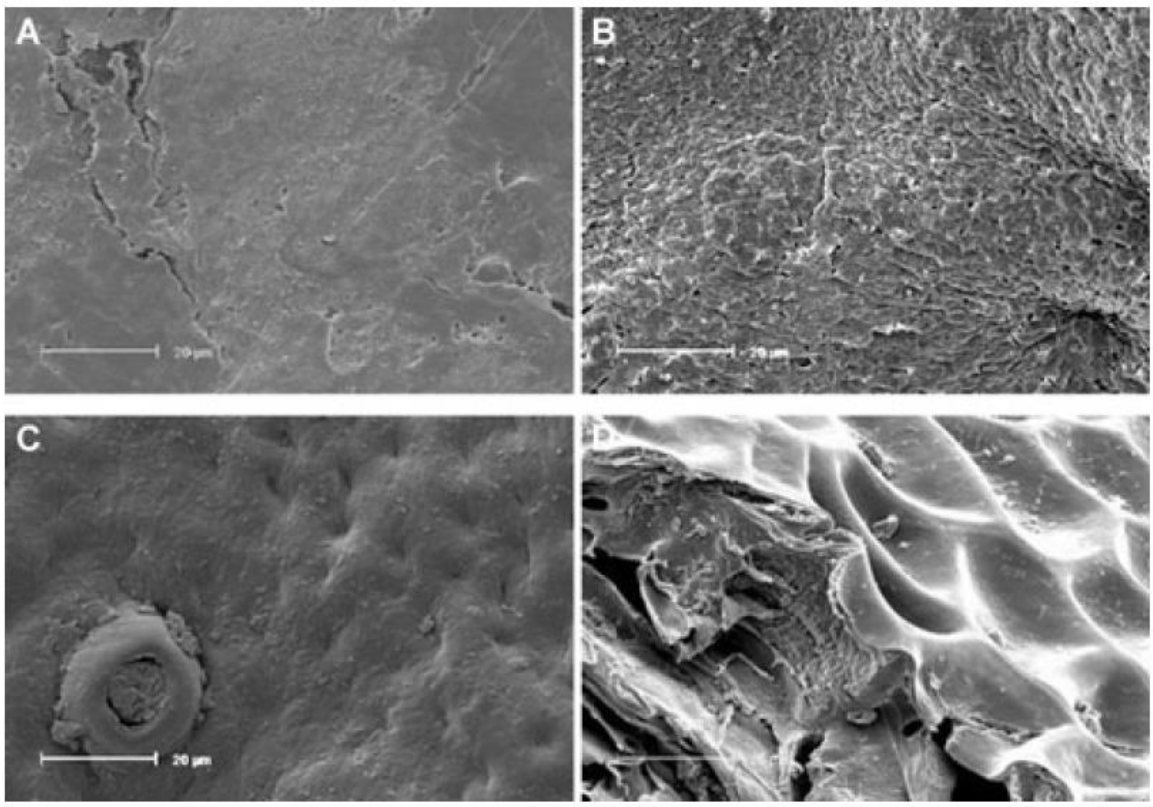

Figure 2.2 Environmental Scanning Electron Microscopy (ESEM) images (x2400) showing the roughness of apple (A), avocado (B), pear (C) and cantaloupe (D) (Wang *et al.*, 2009).

Numerous methods for the elimination of surface bacteria are possible. Popular thermal methods include hot water immersion and steam treatment. Steam treatment is deemed a desirable method for the cleaning of cantaloupe rinds because it is readily diffusible, tissue penetrating and chemically stable leaving no residue (Bezanson *et al.*, 2018). Bezanson *et al.* (2018), found that *L. innocua* surrogate strains employed as benchmarks for *L. monocytogenes* were capable of attachment to the rind of cantaloupes, with two of these strains surviving on the rind for seven days at 7^{o}C. Evidence showed that heat-treated cantaloupes where *Listeria* cells survived had no signs of the proliferation of these cells during post-treatment refrigeration. There was also an absence of culturable *Listeria* from the steam leaving the washer. It should be noted that the efficacy of aerated steam treatment would be greater in isolates that have had no preconditioning to heat stress. Internal pulp of cantaloupe which is exposed, for instance in damaged fruit, has a higher capacity to support the growth of *Listeria*

spp. (Bezanson *et al.*, 2018). This is corroborated by Nyarko *et al.* (2016), who observed that the location of an isolate on the fruit was influential to its survival and proliferation. Bezanson *et al*. (2018) also found that there was an increase in the CFU of *L. innocua* that had colonised stem tissue, but that the intact cantaloupe rind was naturally unfavourable for *Listeria* growth. Interestingly, this was not the case when *L. innocua* was inoculated onto cantaloupes rinds post-heat treatment, as there was significant growth observed (Bezanson *et al.*, 2018). This was largely deemed to be as a result of low numbers of indigenous bacteria (Bezanson *et al.*, 2018), a finding described by Lardeux *et al.* (2015) who mentioned the negative effect that background microbiota in food matrices have on *L. innocua* and *L. monocytogenes* growth..

Investigations on the efficacy of superheated steam (SHS) treatment show promising results for the control of pathogens on melon surfaces. SHS treatment of 200^{o} C for 20 seconds has the potential to reduce microbial pathogens on watermelon surfaces by 5 log reductions, however, in the case of cantaloupes a log reduction of only 1.92-2.23 cfu/cm^2 was achieved. This difference and the reduced efficacy of SHS on cantaloupes was deemed to be as a result of the surface roughness (Kwon *et al.*, 2018). This surface characteristic affords pathogens protected sites where the treatment efficacy is reduced. This is corroborated by Wang *et al.* (2009) who found that increased surface roughness reduced overall microbial reduction as further elucidated in a study by Fransisca and Feng, (2012) where high surface roughness reduced the inactivation properties of bactericidal treatment. This is an important factor in the processing of cantaloupes, regarding cleaning chemical concentrations and times (Kwon *et al.*, 2018).

Bahrami *et al.* (2020) assessed a number of novel and unconventional technologies on their efficacy in reducing *L. monocytogenes* in food products. Of the more extensively researched and promising methods is that of high-pressure processing (HPP). There is significant evidence to support a 5-log reduction of *L. monocytogenes* in certain products through the use of HPP. HPP is performed by mechanically pressurised liquids exerting forces in excess of 100 MPa on a food product (Bahrami *et al.*, 2020). The efficacy with which HPP can be applied to a food product is dependent on some primary factors like temperature, applied pressure, holding time, food matrix composition and properties (pH and a_w), and strain-related sensitivity of *L. monocytogenes* (Bahrami *et al.*, 2020). One considerable drawback to HPP is the potential to sub-lethally injure cells; these bacteria may recover during storage especially if the treatment to the product is not intense enough or if considerable temperature abuse occurs (Balamurugan *et al.*, 2016; Li and Gänzle, 2016; Misiou *et al.*, 2018). Bahrami *et al.* (2020) support this

finding and further highlight the drawbacks of sub-lethally injured cells. Surviving sub-lethally injured cells have the potential to recover during storage, warranting caution with novel technologies and their ability to sub-lethally injure cells.

Postharvest processing is a major contributor in enabling the colonisation and proliferation of *L. monocytogenes* on fresh produce (Hoelzer *et al.*, 2012). In a study by Bezanson *et al*. (2018) there was particular evidence supporting that some post harvesting procedures on whole cantaloupe may promote the growth of *L monocytogenes*, as was observed with *L. innocua*.

L. monocytogenes' ability to proliferate on the rind of cantaloupe has been poorly characterised. The listeriosis outbreak of 2011 in the USA, linked to whole cantaloupe, was found to be as a result of strain serotypes 1/2a and 1/2b, both of which had also been implicated in a febrile gastroenteritis outbreak in non-produce foodstuffs (Lomonaco *et al.*, 2013).

The study found that the strains that resulted in the outbreak had more pronounced growth on the rind of the cantaloupe than in a cantaloupe extract or pulp. This was contrasted by another study which found that *L. monocytogenes* was capable of survival on the rind of cantaloupe but not growth at refrigeration temperatures (Ukuku and Fett, 2002). Lomonaco *et al.* (2013) found limited differences between the adherence capabilities of the strains in the 2011 outbreak. Limited differences between adherence potential between other strains was also noted by Upadhyay (2014).

These findings suggest that the outbreak may have been from stochastic environmental contamination as opposed to an enhanced capacity for adherence or growth on the produce (Martinez *et al.*, 2016). The ability for an organism to adhere to surfaces is influenced by not only the cell surface properties but also by fimbriae and flagella which can aid in surface attachment. Flagella-based movement is considered a requirement for the initial attachment during the formation of biofilms, this is due to the need to overcome interfacial forces (Di Ciccio *et al.*, 2012). Flagella have also been identified as adhesive structures (Di Bonaventura *et al.*, 2008), furthermore, Lemon (2007) was able to elucidate the importance of flagellum-mediated motility in the adhesion and biofilm formation characteristics of *L. monocytogenes* on abiotic surfaces. It was also considered that these outbreak strains may have unique attributes associated with virulence or adherence capability (Martinez *et al.*, 2016). Martinez *et al.* (2016) also found that washing of inoculated fragments resulted in a temporary reduction of *L. monocytogenes* counts of 1.5 to 2 log CFU, with another study finding similar reductions on spot inoculated cantaloupe rind (Upadhyay *et al.*, 2014). Other researchers found that

washing of whole fruit had negligible impact on the amount of *L. monocytogenes* present (Ukuku and Fett, 2002; Ukuku *et al.*, 2012). Conclusively the findings suggested that whole cantaloupe be eaten soon after washing and not stored for more than 72 hours at ambient temperatures (Martinez *et al.*, 2016).

It is important to note that there are also measures consumers can take in order to minimise their risk of exposure to contaminated cantaloupes. These include avoiding cantaloupes with obvious blemishes, washing your hands before handling, scrubbing of the melon and rinsing with water, adequate cleaning of chopping boards and general kitchen hygiene practices (Bowen *et al.*, 2006).

2.3.3 Papaya

Post-harvest controls are also a major influencing factor for the occurrence of *Listeria* on papaya. Most of the research conducted regarding papaya is primarily related to quality control and management. This serves to help interpret the potential avenues for contamination of the fruit. Although *L. monocytogenes* is not typically associated with papaya, there is evidence of its isolation from the fruit (Vahidy *et al.*, 1992 as cited by Raybaudi- Massilia *et al.*, 2013).

Papaya is a fruit crop that typically sees a large amount of post-harvest losses (Azene *et al.*, 2014). As a perishable fruit with a postharvest life of four weeks, it is essential that the papaya which is processed for human consumption is of adequate microbial safety. With the majority of postharvest losses resulting from damage or mechanical injury, the growth of microbes is increased as a result of cut surfaces and can negatively impact the shelf life of the fruit (Sivakumar and Wall, 2013). Papaya pulp is ideal for the growth of *Listeria* (Feng *et al.*, 2015), as it has a neutral pH and nutritional composition high in sucrose, glucose and fructose (Sivakumar and Wall, 2013), all of which are highly favourable for the *Listeria* spp. metabolic function.

Papaya are not typically washed when sold in domestic markets, however, in South Africa they are washed to remove latex and to make the appearance more appealing to consumers; this is typically done using sodium hypochlorite solution (Sivakumar and Wall, 2013). There is also the added benefit of the use of a chlorinated water dip, as it has shown to reduce the microbial count of papaya prior to peeling and cutting (Sivakumar and Wall, 2013). This water becomes a major point for potential cross-contamination as it becomes contaminated with bacteria

present on the unwashed fruit as well as from the debris on the papaya. Water temperature is also considered an important factor for the transfer of bacteria between the papaya and the wash water and must be monitored during post-harvest processing (Sivakumar and Wall, 2013).

Post-harvest procedures like the use of organic acids and ultraviolet (UV)-C have shown potential to reduce *L. monocytogenes* on the surface of papaya. *L. monocytogenes* populations did not change when the fruit was treated with UV-C light (2.88-5.76 kJ/m^2) after being dipped in water or a calcium lactate (1%) and ascorbic acid (0.5%) combination, however, upon the addition of malic acid (0.5-1.5%) significant reduction of the pathogen occurred (Raybaudi-Massilia *et al.*, 2013). Interestingly, malic acid was also shown to be effective in the control of *L. monocytogenes* in melon juice (Raybaudi-Massilia *et al.*, 2009).

Papaya has been previously implicated in outbreaks of *Salmonella* in both Mexico and Australia, showing the role this fruit has as a vector for pathogens (Sivakumar and Wall, 2013). The risks associated with fresh produce are best mitigated by adhering to proper agricultural and postharvest practices. An ideal example of this was the *Salmonella* outbreak in Australia, resultant from washing the fruit with untreated river water. This was considered a contributing factor for the internalisation of pathogens (Sivakumar and Wall, 2013).

Other potential sources for pathogenic contamination are a lack of adherence to good manufacturing procedures (GMPs). These are related to inadequate cleaning and sanitation of packhouses and equipment leading to biofilm formation, poor worker hygiene and other contributing environmental factors like the use of animal manure or water from a contaminated source (Sivakumar and Wall, 2013).

2.3.4 Avocado

Avocado, and in particular avocado pulp and RTE dips like guacamole, have been previously identified as having potential for contamination by *L. monocytogenes* (Iturriaga *et al.*, 2002; Strydom *et al.*, 2013; Chen *et al.*, 2016b; Salazar *et al.*, 2020; Pomeroy *et al.*, 2021). Research regarding the contamination of whole avocado is limited, however, the role of soil and irrigation water, as well as the rough rind of the fruit have been postulated as contributing to the presence of *L. monocytogenes* (Wang *et al.*, 2009; Rodríguez-Garcia *et al.*, 2011; García-Frutos *et al.*, 2020). The role of post-harvest disinfection in increasing the available water on

the surface of the fruit may also enable the survival and proliferation of *L. monocytogenes* and may facilitate the pathogens entry into the pulp via the stem scar (Chen *et al.*, 2016b).

Both avocado pulp and guacamole have been found to support the growth of *L. monocytogenes*, with research suggesting the best method for controlling this organism in these products is by ensuring proper cold chain storage and lowering of the pH (Bill *et al.*, 2014; Ziegler *et al.*, 2018; Salazar *et al.*, 2020). Proper management and monitoring of *L. monocytogenes* in the food processing environment is paramount to better managing the incidence of this pathogen with avocado (Strydom *et al.*, 2016).

2.4 *L. monocytogenes* change from saprotroph to pathogen

L. monocytogenes is one of the best understood Gram-positive intracellular pathogens, largely attributed to the susceptible and manipulatable murine model (Fsihi *et al.*, 2001). Once *L. monocytogenes* enters the host through a vector like a contaminated food product, there are a number of environmental changes which lead to the regulation of the positive regulatory factor A (*prfA*) and the activation of virulence factors (de las Heras *et al.*, 2011). Some of the environmental factors which facilitate the change from saprotroph to pathogen include a drop in pH and available carbon as well as other stresses from the host gut (Fuchs *et al.*, 2012). Although existing theories mention temperature as a primary factor in switching on *prfA*, recent findings have linked carbohydrate source as a key factor in transition to the pathogenic state (de las Heras *et al.*, 2011; Fuchs *et al.*, 2012). As the availability of sugars begins to change once *L. monocytogenes* enters the host cytosol, the organism converts its metabolism from glycolysis to the pentose phosphate pathway (Fuchs *et al.*, 2012; Xayarath and Freitag, 2012). The importance of *prfA* as a survival tool in *L. monocytogenes* cannot be overstressed as it allows the organism to conserve energy in the natural environment by repressing virulence genes (de las Heras *et al.*, 2011; Xayarath and Freitag, 2012). The reverse is true for the survival in a host, represented by the down-regulation of genes crucial to motility when the organism is at 37^{O}C (de las Heras *et al.*, 2011). There is also significant evidence linking the previously mentioned σ^{B} factor to the regulation of genes that transcribe for virulence factors once within the gut (de las Heras *et al.*, 2011).

2.4.1 Listeriosis and the mode of infection in the healthy and immunocompromised

With respect to the pathogenic lifestyle of *L. monocytogenes*, the disease from the infiltration of the bacterium into a host can take several forms but is classified broadly as either being invasive or non-invasive (Camejo *et al.*, 2011). Healthy individuals who are immunocompetent and are exposed to *L. monocytogenes* contaminated foods typically end up with self-limiting and mild gastroenteritis (Drevets and Bronze, 2008; Xayarath and Freitag, 2012). Those most at risk of severe listeriosis are individuals who are part of the high-risk population like the immunocompromised – those undergoing chemotherapy, with HIV/AIDS or transplant patients. As well as the elderly and pregnant women (Bortolussi, 2008) – serious *L. monocytogenes* systemic infections can manifest as meningitis, encephalitis and bacteraemia, resulting in death or infection of the foetus and stillbirth in the case of pregnancy. While the disease listeriosis is not as commonly reported as infections resulting from other food-borne pathogens, it does have one of the highest mortality rates (Bortolussi, 2008; Xayarath and Freitag, 2012).

Listeriosis in humans is most likely as a result of the consumption of contaminated foods (Ooi and Lorber, 2005) which act as a vector for *L. monocytogenes* into the host. Once inside the host there is an incubation period prior to infection which can differ significantly based on the vulnerability of the host (Goulet *et al.*, 2013). The incubation period is generally perceived as quite lengthy and not particularly uniform as seen on analysis of the median time for invasive listeriosis which was determined as eight days, when the data set ranged from one to 67 days. Pregnancy cases had a longer median period of 27.5 days. Central nervous system (CNS) cases had a median time of nine days while bacteraemia was shown to be just two days. Gastroenteritis was 24 hours with a range from six to 240 hours (Goulet *et al.*, 2013).

The different manifestations of listeriosis is primarily a result of *L. monocytogenes'* ability to cross the placental, intestinal and blood-brain barriers (Bécavin *et al.*, 2014). Once the contaminated food enters the gut the bacteria enters the non-phagocytic epithelial cells, facilitated by virulence factor internalins (de las Heras *et al.*, 2011; Fuchs *et al.*, 2012). Once in the cytoplasm the bacteria are protected from any immune-mediated attack and are able to replicate unhindered (Fuchs *et al.*, 2012). Movement within the cytoplasm of infected cells and into adjacent cells is postulated as due to actin polymerisation at the surface of *L. monocytogenes* (Welch *et al.*, 1997; Grenklo *et al.*, 2003; Forsythe, 2007). *L. monocytogenes* is then able to escape the cell through virulence factors that enable lysing of the host cell (Fuchs

et al., 2012) and manifestation of the infection in the blood, CNS and placenta (Forsythe, 2007). New methods for the isolation, enumeration and detection of *L. monocytogenes* are just some of the ways in which management of this pathogen is improving food safety and aiding in the prevention of listeriosis.

2.5 Methods for the isolation and identification of *Listeria* spp. and *L. monocytogenes*

At the forefront of microbial food safety is the detection and identification of foodborne pathogens in a product before it enters the retail market. Potter *et al.* (2012) found that within the US, UK and Republic of Ireland, biological hazards were the second greatest cause for product recalls and contributed to 36% of all recalls between these three countries. Three primary hazards were attributed to the 86% of the total biological recalls, these being *L. monocytogenes*, *Salmonella* spp. and *Escherichia coli*. *L. monocytogenes* was the second-most common cause for the above-mentioned biological recalls (Potter *et al.*, 2012). Although *Salmonella* was identified as the primary biological hazard associated with fruit, vegetables and salads (Potter *et al.* 2012), there is significant evidence indicating the prevalence of *L. monocytogenes* on a range of fruit (Feng *et al.*, 2015; Spadafora *et al.*, 2016; Bezanson *et al.*, 2018). Fresh and fresh-cut produce are rapidly growing market segments in developed countries like the USA (Niemira and Fan, 2012). The notion that as human diets continue to change, so too will our susceptibility to foodborne pathogens is conceivable and requires a response to this increased risk by developing innovative surveillance of the pathogens present in the food we produce and consume (Batt, 2016). As a minimally-processed RTE product, the microbial safety of fresh-cut produce is of particular importance, as there is no additional treatment post-processing – or similar major sterilising procedure like a heating step – to ensure elimination of potential pathogens (Hong *et al.*, 2014). This places a huge degree of importance on the monitoring procedures, sampling and detection methods of the food business operator (FBO). There are a number of current and emerging methods available for the identification, typing and genetic profiling of *L. monocytogenes* and other *Listeria* spp., as well as technical outcomes of swabbing and detection investigations.

2.5.1 *L. monocytogenes* detection and culturing methods

Culture methods for *Listeria* spp. have long been an important method for the monitoring of food safety and acceptability. These methods are typically sensitive, cost-effective and allow for detection and enumeration (Churchill *et al.*, 2006). There are a range of available and accredited methods for the detection of *L. monocytogenes* with many of them differing in minor details having been optimised over time (Zunabovic *et al.*, 2011). The success of a culturing method is largely dependent on the initial number of organisms, the state they are in, the selectivity of the media and the time and conditions of incubation (Beumer and Hazeleger, 2003 as cited by Churchill *et al.* 2006). Currently the most common methods for the detection of *L. monocytogenes* include: ISO 11290-1; NGFIS; Cold Enrichment; NMKL method no. 136; USDA-FSIS for meat and poultry products; and the Food and Drug Administration (FDA) bacteriological and analytical method (BAM) for seafood, dairy, fruit and vegetables (Gasanov *et al.*, 2005; Churchill *et al.*, 2006; Loncarevic *et al.*, 2008; Gnanou Besse *et al.*, 2019).

Today's culturing methods are made specific and effective for the determination of *Listeria* spp. as a result of the advent of chromogenic agar. The first effective chromogenic agar available commercially was ALOA (Agar *Listeria* according to Ottaviani and Agosti). Capable of distinguishing between pathogenic and non-pathogenic *Listeria* spp. due to the activity of phosphatidylinositol-specific phospholipase C (PI-PLC) and to a lesser extent phosphatidylcholine phospholipase (PC-PLC). Chromogenic media are separated into two categories with the first using cleavage by PI-PLC of L-α-phosphatidylinositol. This results in a white ring of precipitation zone around the pathogenic colony, combined with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside for detection of β-D-glucosidase, which occurs in all *Listeria* spp. resulting in turquoise colonies. Media that are part of this category include ALOA and *Brilliance Listeria* Agar (BLA) (Formerly Oxoid Chromogenic *Listeria* Agar (OCLA)) as well as Harlequin™ *Listeria* Chromogenic Agar (Neogen) (Reissbrodt, 2004).

The second group of media utilizes 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate for the identification of pathogenic *Listeria* colonies which appear as a blue-turquoise colour, whilst non-pathogenic colonies appear a white colour. *L. monocytogenes* is further characterised through a lack of xylose fermentation. The most noteworthy medium in this category is the widely used RAPID'L.mono™ Medium (Bio-Rad) (Reissbrodt, 2004). One of the other common media used, but which is unable to distinguish pathogenic from non-pathogenic species (Lauer *et al.*, 2005; Zunabovic *et al.*, 2011), is Polymyxin Acriflavine

Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar. PALCAM agar differentiates *Listeria* spp. from other bacteria based on two systems: i.) the hydrolysis of esculin in the presence of ferric iron, which causes blackening of the medium by *Listeria* spp., and ii.) mannitol fermentation in the presence of phenol red. Mannitol fermentation is not possible by *Listeria* spp. and its presence in conjunction with phenol red, a pH indicator, allows for the indication of contaminating bacteria like *Enterococci* on the agar visualised through the change of medium colour from red to yellow as acidic products begin to accumulate. Black haloes are the definitive indication of *Listeria* spp. (van Netten *et al.*, 1989; Churchill *et al.*, 2006).

In fact, PALCAM agar, included in these former reference methods, cannot distinguish *L. monocytogenes* from other *Listeria* spp. colonies. In these methods, five typical colonies had to be confirmed per plate, and may not have been *L. monocytogenes*. This dramatically decreased method sensitivity (Gnanou Besse *et al.*, 2019).

Concerns regarding the growth of non-*Listeria* bacteria on selective media has been previously noted, with *Staphylococcal* species with β-D-glucosidase activity (Dakić *et al.*, 2005; Angelidis *et al.*, 2015), as well as a number of species within the genus *Bacillus* and *Enterococcus* capable of growing on chromogenic agars (Angelidis *et al.*, 2015).

As a result of the scope of this study and its applicability in the South African context only ISO-approved methods used by the FBO will be discussed and evaluated. In this instance the specific method utilised for detection and enumeration is ISO 11290:2017.

2.5.2 ISO method 11290 for the detection and enumeration of *L. monocytogenes*

There are of course also a number of drawbacks to the classic culturing methods and techniques employed by the food industry, most notably time constraints and necessary labour required (Churchill *et al.*, 2006). This is no different to the ISO approved method for the detection of *L. monocytogenes* (Dalmasso *et al.*, 2014). This method relies on a two-stage enrichment procedure. This is done due to the relatively low numbers of *L. monocytogenes* present in the environment and food samples and the high amount of competing microflora present in samples (Bruhn *et al.*, 2005; Auvolat and Besse, 2016). The two-stage enrichment procedure has come under some scrutiny due to the inhibitory effects of some selective agents on *L. monocytogenes*

(Jacobsen, 1999; Gasanov *et al.*, 2005). The current standard for the isolation and enumeration of *L. monocytogenes* is a 2017 revised standard of ISO 11290-1&2:2004 (Gnanou Besse *et al.*, 2019). This is the only ISO-approved method for the detection and enumeration of *L. monocytogenes* in food and environmental samples and is outlined in Table 2.3, and looks to improve on the drawbacks of the previous procedure by making some significant changes (Gnanou Besse *et al.*, 2016, 2019; Rollier *et al.*, 2019).

Table 2.3 Changes and differences between ISO 11290:2004 and 11290:2017

Part of the procedure	Differences		Motivation
	ISO 11290-1&2:2017	ISO 11290-1&2:2004	
Primary enrichment	Incubation time in half-Fraser broth is 25 h ± 1 h	Incubation time in half-Fraser broth is 24 h ± 2 h	To improve growth of stressed *L. monocytogenes* cells
Secondary enrichment	Reduced by half so that the incubation time is 24 h	Incubation time of 48 h	Similar strain recovery at 24 and 48 h with no impact on the recovery of *L. monocytogenes*
Enrichment media	Both half and full Fraser broth may be refrigerated for a maximum of 72 h prior to transfer or isolation on selective agar	No refrigeration	Increase selective pressure on non-psychrophilic bacteria
CAMP and catalase test	Optional for *L. monocytogenes*	Required	Improved methods for detection of virulence and catalase activity
Haemolysis and CAMP test	Use of defibrinated sheep, calf or bovine blood in blood agar. For haemolysis test inoculation of blood agar by stabbing or streaking if positive at purification step	Only defibrinated sheep blood for blood agar.	To enhance accessibility and practicability of testing materials.
Microscopy	Mandatory, however, the use of an agar which distinguished pathogenic *Listeria* spp. is used	Mandatory	To ensure and confirm colony identification
Initial suspension	In addition to the diluents described in the previous standard, one may use half-Fraser broth with selective agents and all diluents described in ISO 6687. The 1 h resuscitation step was removed	Use of Buffered Peptone water and half-Fraser broth without selective agents and a 1 h resuscitation step at ambient temperatures	Mitigate enrichment bias
Selection of plates	Maximum 150 colonies per plate, however in the case of mixed cultures and/or overlapping halos the plates selected should be fewer than 100 characteristic *Listeria* spp. colonies	Maximum 150 colonies per plate	Avoid the selection of potentially contaminated plates or overgrown plates

(Gnanou Besse *et al.*, 2016, 2019; Rollier *et al.*, 2019)

Although there are a number of researchers who argue that a primary enrichment step in half-Fraser broth is generally sufficient for the detection of *L. monocytogenes* (Loncarevic *et al.*, 2008; Oravcová *et al.*, 2008; Gnanou Besse *et al.*, 2016), it cannot be stressed enough that secondary enrichment in full-Fraser broth results in a greater number of *L. monocytogenes* positive samples (Gnanou Besse *et al.*, 2019). Particularly for samples low in *L. monocytogenes* or with high amounts of competing microflora (Loncarevic *et al.*, 2008; Auvolat and Besse, 2016; Gnanou Besse *et al.*, 2019), such as environmental samples or other foods which may contain stressed cells. There are some important considerations regarding secondary enrichment, as some *L. monocytogenes* may not be detected due to the overgrowth of other *Listeria* spp. (Bruhn *et al.*, 2005; Gnanou *et al.*, 2010; Zilelidou *et al.*, 2016).

Other methods not accredited by ISO are permissible based on the assessment of the governing body in question. In various countries throughout Europe, the competent authority allows the network of accredited laboratories to use alternative methods, even for official controls, if they have been validated according to the EN ISO 16140 standard, and certified by a third party (Auvolat and Besse, 2016). Laboratories are also not all restricted to a particular set of media and within ISO 11290-1&2:2017 there are a number of accepted agar and enrichment media (Gnanou Besse *et al.*, 2019; Rollier *et al.*, 2019).

Ultimately ISO 11290-1&2:2017 was deemed efficient for the detection and enumeration of *L. monocytogenes* in foods and food-related environments (Gnanou Besse *et al.*, 2019; Rollier *et al.*, 2019). Additionally the value of detection was satisfactory for one cell in 25 g food sample (Gnanou Besse *et al.*, 2019). The specificity of the method has been improved since 2004 with the introduction of chromogenic agar, most notably ALOA, which distinguishes *L. monocytogenes* from other *Listeria* spp. and allows enhanced confirmation of *L. monocytogenes* (Rollier *et al.*, 2019).

2.5.3 Enrichment bias

Although *Listeria* and *L. monocytogenes* are widely distributed in nature, they occur in comparatively low numbers to other microorganisms. It is for this reason that a selective enrichment step is included in almost all methods for the detection of *Listeria* spp., in order to amplify the small amount of cells for detection (Oliver *et al.*, 2007).

The same is the case when detecting *Listeria* spp. from food matrices or food production environments (Bruhn *et al.*, 2005). There are a number of controversial findings with regard to the enrichment of *Listeria* spp. and in particular *L. monocytogenes* due to the propensity for false negatives, overgrowth and lineage or subtype bias (Oliver *et al.*, 2007).

Considering that foods can be contaminated with more than one strain of pathogen, it is important to academics and the food industry to accurately determine the presence of a strain in a food sample, in order to effectively manage outbreaks and food safety (Zilelidou *et al.*, 2016). However, the competitive nature of *L. monocytogenes* during selective enrichment, as well as the competitive nature of background microbiota and other *Listeria* spp. (Gasanov *et al.*, 2005; Oravcová *et al.*, 2008; Zilelidou *et al.*, 2016), make the accurate determination of the *L. monocytogenes* strains present in food challenging. As a result, numerous strains of *L. monocytogenes* have been implicated in a single listeriosis outbreak (Zilelidou *et al.*, 2016).

There are several factors which will impact the concentration of *L. monocytogenes* present at the end of incubation in half-Fraser broth. The number of initial viable cells, the physiological state of the cells in the contaminated food product, the growth properties of the strain and the nutritional properties of the enrichment broth and the background microbiota are some of the common and major influencing factors on *L. monocytogenes* concentration following enrichment (Augustin *et al.*, 2016). However, mounting research to better understand enrichment bias and overgrowth within the *Listeria* genus have highlighted a number of smaller more nuanced influential factors which have not been previously considered. For the purpose of understanding enrichment bias and overgrowth, the factors affecting *L. monocytogenes* will be divided into i.) growth rate; ii.) lineages; and iii.) selective agents.

2.5.3.1 Growth rate and quorum sensing

One of the most highly-hypothesised microbial attributes affecting enrichment bias is the growth rate of isolates within the enrichment media. This is noted specifically with regards to *L. innocua*, which is often isolated with *L. monocytogenes* and is common in food production and urban environments (Orsi and Wiedmann, 2016). One research study which focused on enrichment bias in University of Vermont Medium (UVM), found that *L. innocua* exhibited faster growth rates during enrichment in UVM than *L. monocytogenes* (Bruhn *et al.*, 2005), resulting in false negatives due to overgrowth of *L. innocua*. Gnanou, *et al.* (2010) shows findings that support the higher growth rate for *L. innocua* in both the full and half-Fraser

broths. In half-Fraser broth the overgrown species (*L. monocytogenes*) was nearly half the number of the dominant strain (*L. innocua*). In full-Fraser broth the overgrown species was only 10% of the total number of the dominant species, highlighting the higher growth rate of *L. innocua* as well as the potential inhibitory effect of the selective agents contained in the Fraser enrichment broth.

However, a study by Cornu *et al.* (2002), found that there was no observed significant difference between *L. innocua* and *L. monocytogenes* growth rates in both selective and non-selective broths, although full-Fraser broth was considered the least advantageous for the growth of *L. monocytogenes* as alluded to by Gnanou *et al.* (2010).

Acriflavine, a selective agent in enrichment media to suppress the growth of other Gram-positive bacteria, was more inhibitory for *L. monocytogenes* compared to *L. innocua* (Beumer *et al.*, 1996). Cornu *et al*., (2002) found, in the assessment of full-Fraser broth enrichment, that there were no observed differences in generation or lag times between several different strains of *L. monocytogenes* and *L. innocua* in non-selective media, potentially supporting the notion that overgrowth or the apparent growth rate difference may be as a result of the selective agents present in the enrichment media.

The most telling observation, with regards to microbial growth as an influencer on enrichment bias, is the propensity for the initial dominant specie to remain dominant throughout the enrichment process. Carvalheira *et al.* (2010) found that the inhibition of *L. monocytogenes* was correlated with high initial numbers of *L. innocua* and suggested that bacterial population management may be facilitated by the quorum sensing phenomenon. Quorum sensing is a cellular communication system in which bacteria use the production and detection of extracellular chemicals called autoinducers to monitor cell population density and respond accordingly (Ng and Bassler, 2009). The dominant strain in the sample would persist as the dominant strain during the enrichment process. It was observed that simultaneous cessation of growth would occur when the dominant species reached its stationary phase (Gnanou Besse *et al.*, 2005). This was described by Ross *et al.* (2000) as the “Jameson Effect” which typically results from either the competition for a nutritional source or the build-up of toxic metabolites (Ross *et al.*, 2000). However, Cornu *et al.* (2002) found that an *L. welshimeri* strain had reduced growth rates in a co-culture with *L. monocytogenes* than in a monoculture. This finding supports the notion that growth inhibition is primarily linked to the accumulation of toxic metabolites more than competition for nutrients.

The representation of all subtypes in their appropriate density within a food sample is a vital part of epidemiological investigations (Bruhn *et al.*, 2005). One example of the overgrowth which can occur in classic culturing and detection methods is with *L. innocua* (Oravcová *et al.*, 2008). This species of *Listeria* is common in food, however, because of its potential to grow at a faster rate than *L. monocytogenes* in UVM there may be overgrowth of *L. innocua* causing *L. monocytogenes* to be overlooked, resulting in false negative outcomes (Bruhn *et al.*, 2005). Overgrowth of *L. monocytogenes* by *L. welshimeri* and *L. seeligeri* during enrichment was also observed by Dailey *et al.* (2014, 2015). This emphasises the potential for false negatives, as these two non-pathogenic species of *Listeria* are often found in the environment. Dailey (2014, 2015) ultimately concluded that the observed overgrowth was as a result of growth parameters, like the reduction in nutrients or pH change.

The growth rate of bacteria is impacted by their physiological state and one could say with confidence that contaminated foods have bacteria which have been exposed to a number of stresses (Gnanou Besse *et al.*, 2005). In fact, Gnanou Besse *et al.* (2005) determined cell stress to be a greater determining factor in strain growth than medium composition or incubation temperature. That is not to say that incubation temperature or medium composition do not play a role, as the selective components in Fraser broth delay the repair of heat-injured cells, and result in an extended lag phase (Silk *et al.*, 2002).

The medium can further affect growth bias as a result of its structure like thickness, mainly related to its effect on nutrient availability and diffusion (Zilelidou *et al.*, 2016). This was supported when outgrowth of *L. monocytogenes* by other *Listeria* spp. was reduced following the addition of agar to an enrichment broth. This is because cells which are immobilised on agar are subject to diffusion limits for both nutrients and metabolites. The addition of something like agar to an enrichment broth reduces bulk transfer through the medium, and hence allows the slower growing strain to increase in population (Gnanou Besse *et al.*, 2010) (although the addition of agar to enrichment broths is not practically realistic).

One of the final considerations regarding the effect of growth rate on *Listeria* overgrowth is the incubation time and temperature. Although temperature is optimised to reduce processing time and for optimal growth of the organisms, the incubation period has shown potential advantages in isolating *L. monocytogenes* when altered.

A twostep procedure has an increased likelihood to enhance the overgrowth phenomenon. Meanwhile Loncarevic and Oravcová (2008) have described the detection of *L. monocytogenes*

following the primary enrichment in half-Fraser, however the secondary enrichment in Fraser broth will ultimately result in more cells, particularly when the food sample contains low levels of *L. monocytogenes* or high levels of competing microflora (Loncarevic *et al.*, 2008). However, some *L. monocytogenes* may be lost due to overgrowth of other *Listeria* species (Loncarevic *et al.*, 2008). It was these findings and those of Gnanou Besse *et al.* (2005) that highlighted the potential to reduce the enrichment procedure by 24 hours.

2.5.3.2 Environment

Bias between *L. monocytogenes* strains as a result of their environment has been a consideration, especially when the contaminated food is used as a vehicle for the organism in the enrichment broth (Zilelidou *et al.*, 2016). Even though selective enrichment has made isolation easier, the genetic and phenotypic differences between serotypes could extend to the enrichment process, a high-stress selective environment that demands competing with background microbiota from the food product along with a number of selective agents (Gorski *et al.*, 2006). *L. monocytogenes* bias in food products has been hypothesised in a study by Porto *et al.* (2003), whereby five *L. monocytogenes* strains from lineages I and II were inoculated onto frankfurters. This resulted in no significant difference in the frequency of detection for the five strains on modified Oxford agar after 28 days. However, there was a higher percentage of lineage II strains isolated than lineage I, with the highest number of lineage II (serotype 1/2a) isolated after 90 days. This study elucidated the lack of significant short-term competitive bias between lineages I and II, but also the competitive advantages of serotypes within the same lineage. This was seen when there was a difference between the frequency with which some lineage II isolates where recovered compared to others.

Ultimately Porto *et al*. (2003) were able to provide evidence which showed that the source of an *L. monocytogenes* isolate is not always indicative of its specific adaptive abilities; this is supported by the better growth performance in a vacuum-sealed frankfurter observed by an environmental isolate than by isolates from frankfurters, other deli sausages and human patients. Conversely Zilelidou *et al.* (2016) found that *L. monocytogenes* isolates recovered from ground pork had a competitive advantage over non-pork isolates in minced-meat mixed enrichments.

2.5.3.3 Selective agents

The role selective agents play in selecting for organisms in enrichment broths has been well documented and is significant, with a summary of these effects outlined in Table 2.4. If the selective agents used in enrichment skew the results of swabbing, then there will be a considerable disparity, as seen with *L. monocytogenes*, in terms of their representation in both environmental and food swabs, and clinical isolates. This can greatly impact the results of epidemiological studies following outbreaks and can make tracing the source of an outbreak especially challenging. This, for the most part, is one of the major justifications for the use of a primary enrichment step, in order to avoid false negatives that may arise due to the strong inhibitory effect of selective enrichment substrates (Supanivatin *et al.*, 2012).

The experiment most referred to with regards to the effect of selective agents and their impact on enrichment outcomes is by Bruhn *et al.*, (2005). UVM as a medium contains a number of selective agents. Nalidixic acid is used in UVM to ensure Gram-negative bacteria are suppressed and only has a minor effect on *L. monocytogenes* growth at the highest threshold concentration, however, other selective agents like lithium chloride (LiCl) have been shown to inhibit Gram-positive bacteria. LiCl reduces the growth of *L. monocytogenes* by as much as 50% when added to an enrichment medium (Jacobsen, 1999). Another selective substrate that has adverse effects on *L. monocytogenes* growth and recovery is acriflavine; although acriflavine is effective in reducing both background and competitive microbiota it also has a negative impact on the lag and generation time of *L. monocytogenes* (Beumer *et al.*, 1996). The findings in the study by Jacobsen (1999) corroborated those of Beumer (1996) in that acriflavine had a negative effect on the recovery of *L. monocytogenes* from enrichment media.

Table 2.4 Role of different substrates used in selective media on *L. monocytogenes* survival

Selective substrate	Function	Major enrichment media	Effect on *Listeria*	Reference
Acriflavine	Inhibition of Gram-positive bacteria through inhibition of RNA synthesis and the production of mitochondria	UVM; Fraser broth and supplement; *Listeria* enrichment broth (LEB); Brilliance *Listeria* supplements and agar	Strain related inhibition of *L. monocytogenes*, interference with colony colours on chromogenic media, concentration influences lag and generation times.	(Beumer et al 1996); (Jacobsen, 1999); (Bruhn *et al.*, 2005) (Beumer and Curtis, 2003)
Fosfomycin	Antibiotic for both Gram-positive and negative	*Listeria* selective supplement	Selectively inhibits *L. ivanovii*, *L. seeligeri*, *L. welshimeri*	(van Netten *et al.*, 1989)
Lithium chloride	Inhibits Gram-positive and slightly reduces Gram-negative bacteria	Fraser supplement and broth; Brilliance *Listeria* agar; PALCAM supplement and agar base; ALOA	Strong inhibition of *L. monocytogenes*	(Cox et al 1990) (Jacobsen, 1999)
Nalidixic acid	Inhibition of Gram-negative bacteria. Inhibits DNA synthesis of cells	Fraser supplement and broth; UVM; LEB; Brilliance *Listeria* enrichment and differential supplement; ALOA	Minor inhibition at concentration threshold	(Jacobsen, 1999) (Beumer and Curtis, 2003)
Polymyxin B	Inhibition of Gram-negative bacteria	Fraser supplement and broth; LEB; Brilliant *Listeria* Agar; Chromogenic *Listeria* Agar etc.	Weak inhibitory effect against some strains of *L. monocytogenes*	(Jacobsen, 1999)
Cycloheximide	Prevents growth of yeasts and moulds	*Listeria* repair broth (LRB) Oxford agar		(Beumer and Curtis, 2003)
Phenyl ethanol	Inhibit growth of Gram-negative bacteria	Lithium chloride-phenylethanol-moxalactam (LPM) agar	Inhibition of *L. monocytogenes*	(Van Netten 1988) (Jacobsen, 1999)

2.6 Listeriosis and the risk it plays to consumers

Human exposure to *L. monocytogenes* is, as previously stated, primarily due to the consumption of a contaminated food product. Although contamination of foods in processing plants is a greater risk for human exposure than contamination closer to consumption, like within the home (Ivanek *et al.*, 2007), it is crucial that consumers are aware of the risks and are competent with regards to basic food safety. This is because of the ubiquity of the organism and its ability to grow in refrigerated foods (Forsythe, 2007), especially with RTE products which require little to no consumer processes that may reduce or eliminate microbes, like heating (Scholliers, 2015). Consumption of a small amount of *L. monocytogenes* on a contaminated food is not uncommon (Valderrama and Cutter, 2013). It is for this reason that significant research has focused on consumer awareness and communication strategies to improve food handling and consumer knowledge of potential risk factors.

In the context of convenience foods – RTE and easy-to-prepare (ETP) meals – there has been a growing demand from consumers for their availability in the retail space. The growth in academic research related to these products has also increased significantly over the course of the last 50 years, with a major surge since 2000 (Scholliers, 2015). This reported increase is corroborated by the increase of convenience meals in the food market (Scholliers, 2015). An example of this progressive change to convenience foods, like RTE meats, is reflected in the weekly consumption of products like lasagne, kebabs and similar products in the United Kingdom (UK) (Scholliers, 2015). Some of the factors influencing the rise of RTE products include the growing role women play in the workforce, lack of time as well as cooking skills, diverse changes in product development and new technology in the home kitchen (Sheely, 2008; Brunner *et al.*, 2010).

Many different methods have been investigated for the communication of the potential risks *L. monocytogenes* poses. Some commentary surrounding the understanding elderly consumers have, has surprising findings. Evans and Redmond (2015) found that adult consumers older than 60 years, from the UK and South Wales, were generally not aware of *L. monocytogenes* as a significant foodborne pathogen. Only 88% (n=100) of their study were capable of naming at least one pathogen associated with a foodborne illness and only 20% named *L. monocytogenes*. However, when prompted, a far greater proportion did recognise *L. monocytogenes* – nearly 87%. The sample group may have been conscious of *L. monocytogenes*, but awareness of the food products normally associated with this pathogen was found to be less than ideal, as 55% of the sample group were unable to identify a food product that they knew of as a risk. More concerning is how not one individual from this study identified themselves as being part of the listeriosis-vulnerable population, instead there was a greater

awareness (44%) of the risk pregnant women are at of contracting listeriosis. This is in line with the general consensus that knowledge regarding this pathogen, even when considering the significant attention it has received, is unacceptably low (Maia *et al.*, 2019). The high awareness of the risk posed to pregnant women may well be contributed to the higher degree of broad risk communication strategies to this affected population group (Kendall *et al.*, 2017). This, as well as pregnant women actively being informed and seeking information regarding listeriosis are contributing factors. The internet has been identified as a good source of information for *Listeria* and listeriosis (Maia *et al.*, 2019). Perhaps the most prevalent outcome of recent studies is that elderly populations are under the risk. This is mainly due to the pretence that because they maintained their food handling practices at home and have not contracted a serious foodborne illness, they can continue to maintain these practices even when they become more at risk of contracting a life-threatening illness like listeriosis (Evans and Redmond, 2016). This is especially pertinent as the elderly are not generally at a high risk of contracting listeriosis from eating high-risk foods, as is the case for pregnant women (Mateus *et al.*, 2014), but rather from poor food-handling practices and consumption of food past its "best before" date (Maia *et al.*, 2019).

In noting the general lack of knowledge the older adult consumer group tends to have regarding *L. monocytogenes*, Evans and Redmond (2015) concluded that there was no significant relationship between consumer knowledge and actual behaviour. This is supported by the resistance older adult consumers have to changing their domestic food handling practices. Eating food that is past the best before date, not ensuring their refrigerators are at the correct temperature as well as other routine behaviours which can increase the risk factors associated with listeriosis are just some examples.

Similar trends with regards to the gap in listeriosis food safety and consumers' willingness to change their practices to mitigate risk is also seen in pregnant women. As reported by Maia *et al.* (2019), of the 78.9% of women aware of *L. monocytogenes*, only 28.9% reported not eating high-risk foods.

There are significant economic impacts as a result of this preventable food-borne disease. The consequences in an outbreak can be far-reaching and may not only affect the lives of the infected and their families, but can be catastrophic for the food industry and global economies (Todd, 1987; Scharff, 2011; Olanya *et al.*, 2019).

2.7 Conclusion

Within the body of research referred to, there are significant findings and information regarding the ability of *L. monocytogenes* to survive in the environment, the processing facility and within the human host. *L. monocytogenes*' modes of infection and ability as an intracellular pathogen, especially

with regards to the roles of different virulence factors is well documented. Furthermore, the role of these virulence determinants and the transfer of other genes amongst *Listeria* species is an area of continued research. There are gaps in understanding with regards to the link between the natural environment and the processing environment, and if *L. monocytogenes* can proliferate equally in both. However, there is a significant amount of research regarding the different environmental factors and their influence on the distribution of species within the *Listeria* genus. The role of fresh fruit and its potential as a vector for this organism is documented for some fruit and regions, but the relevance and applicability of these findings in the South African food chain is in no way guaranteed. This also represents a gap in understanding of *L. monocytogenes*' ecology and requires definite outcomes specific to South Africa for a comparison to other geographical locations and global findings. Furthermore, the potential methodology for the isolation and detection of *L. monocytogenes* remains imperfect, with potential for changes to improve accuracy and reliability, thus improving food safety and mitigating the risk of *L. monocytogenes* outbreaks.

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Chapter 3: The occurrence of *L. monocytogenes* in the RTE prepared-fruit supply chain in South Africa

3.1 Abstract

L. monocytogenes is a ubiquitous environmental pathogen capable of causing the foodborne illness listeriosis. Its presence in ready-to-eat (RTE) prepared-fruit products is of serious concern, as these products cannot undergo heat sterilisation due to the negative effects on quality and sensory attributes. Regular monitoring and management of the pathogen in both the agricultural and food processing environment is therefore crucial. Pre-harvest contamination was found to be exacerbated by environmental factors, whilst certain niches within food processing facilities like the processing environment and raw materials were identified as facilitating the growth and survival of this pathogen. This study assessed the presence of *Listeria* species and *L. monocytogenes* at melon farms in two distinct geographical locations in South Africa (Limpopo and Eastern Cape provinces) as well as interpreted the sources of *L. monocytogenes* and associated lineage types at a prepared-fruit processing facility in Gauteng where fruit from farms in both regions were processed. Weather was shown to have played a role in facilitating the dissemination of *Listeria* spp. in the agricultural environment. *L. monocytogenes* isolates from the prepared-fruit processing environment, raw materials and final product were identified as lineage I (n=16) and lineage II (n=33) through PCR-RFLP typing. Most isolates from the environment and raw materials were lineage II and the more virulent lineage I isolates predominated in final products. The occurrence and distribution of these two lineages within the food processing environment raises questions regarding the efficacy of management practices, whilst also characterising the potential sources of contamination.

3.2 Introduction

Listeria monocytogenes is a rod-shaped, Gram-positive, facultative anaerobic foodborne pathogen that is ubiquitous and capable of survival in both the natural and industrial environments (Weis and Seeliger, 1975; Gandhi and Chikindas, 2007; Ivanek *et al.*, 2006; Di Ciccio *et al.*, 2012; Orsi and Wiedmann, 2016). This is due to this organism's ability to survive at temperatures as low as 0ºC (Goldfine and Shen, 2007), in salt concentrations up to 10% (Ribeiro *et al.*, 2006) and pH ranging

from 4.7-9.2 (Zunabovic *et al.*, 2011). *L. monocytogenes* is not only an effective saprotroph in the environment but is also a prolific foodborne-pathogen capable of causing listeriosis.

Listeriosis is a foodborne disease which can take several forms but is classified broadly as either being invasive or non-invasive (Camejo *et al.*, 2011). Healthy individuals who are immunocompetent and are exposed to *L. monocytogenes* contaminated foods typically end up with self-limiting and mild gastroenteritis (Drevets and Bronze, 2008; Xayarath and Freitag, 2012). Individuals who are immunocompromised, as well as the elderly and pregnant women (Rivero *et al.*, 2003; Bortolussi, 2008) – suffer systemic infections which manifest as meningitis, encephalitis and bacteraemia, resulting in death or infection of the foetus and stillbirth in the case of pregnancy (Disson *et al.*, 2008). While the disease listeriosis is not as commonly reported as infections resulting from other food-borne pathogens are, it has one of the highest mortality rates (Bortolussi, 2008; Xayarath and Freitag, 2012).

South Africa recorded the largest outbreak of listeriosis in early 2017 which was only traced back to ready-to-eat processed meat products in March 2018 as cases of infection continued to increase until July 2018 (Tchatchouang *et al.*, 2020). The extent of the listeriosis outbreak was 1060 confirmed laboratory cases and more than 200 deaths (Tchatchouang *et al.*, 2020), with the actual number of cases considered to be far greater (de Noordhout *et al.*, 2014). The high number of patients with HIV/AIDS in South Africa and resultant immunocompromised community remains a concern for future listeriosis outbreaks (Karim *et al.*, 2009).

Research on the occurrence of *Listeria* spp. and *L. monocytogenes* in the South African food environment is limited, with the listeriosis outbreak in 2017 prompting investigation into better monitoring and managing of the pathogen within the South African food supply chain (Boatemaa *et al.*, 2019; Tchatchouang *et al.*, 2020; Thomas *et al.*, 2020).

The ability of *L. monocytogenes* to change from robust environmental saprotroph to human pathogen is achieved through the expression of a number of genetic virulence determinants, but none more so than those controlled by the transcription regulator PrfA (de las Heras *et al.*, 2011). The genes involved in *L. monocytogenes*' ability to manifest listeriosis, are primarily: *inl*AB, enabling the internalisation and invasion of host cells (Orsi *et al.*, 2011); *hpt* which is attributed to replication in the cytosol; and six genes encoded by the PrfA-dependent gene cluster (Vázquez-Boland *et al.*, 2001; de las Heras *et al.*, 2011).

PrfA-dependant gene cluster, also referred to as *Listeria* pathogenicity island 1 (LIPI-1), is key for *L. monocytogenes* to manifest listeriosis (Vázquez-Boland *et al.*, 2001; Freitag *et al.*, 2009; Maury *et al.*, 2017). Virulence in *L. monocytogenes* is mainly regulated by the genes *prf*A, *plc*A, *plc*B, *mpl*,

*act*A and *hly*. Phospholipases PlcA and PlcB, as well as the pore-forming *hly*A encoded listeriolysin O (LLO), are crucial in the lysing of intracellular phagocytic vacuoles. The *hly*A gene is of particular importance as a target for genetic confirmation of *L. monocytogenes* through polymerase chain reaction (PCR) (Gasanov *et al.*, 2005; de las Heras *et al.*, 2011; Law *et al.*, 2015).

Within the species *L. monocytogenes* there are four distinct genetic lineages based on phylogeny (Haase *et al.*, 2014; Orsi and Wiedmann, 2016). Lineages III and IV are uncommon and are typically isolated from animal cases of listeriosis (Orsi *et al.*, 2011), whilst the majority of *L. monocytogenes* belong to lineages I (1/2b, 3b, 3c, 4b) and II (1/2a, 1/2c, 3a) (Haase *et al.*, 2014). Lineage I isolates are more often implicated in human listeriosis outbreaks and clinical cases due to their virulence, possessing low genetic diversity and recombination rates, whilst lineage II isolates are frequently recovered in food and environmental samples and possess high genetic diversity and recombination rates (den Bakker *et al.*, 2010; Orsi *et al.*, 2011; Haase *et al.*, 2014). Differences in the lineages can be easily determined through the use of restriction fragment length polymorphisms (RFLPs) resulting from restriction enzyme digestion of their *hly*A gene amplicons (Rip and Gouws, 2020).

The occurrence of *L. monocytogenes* in food products is of serious concern due to the ability of the organism to persist at low temperatures (0^{o}C) allowing it to grow in refrigerated products or products that rely on the cold chain to ensure consumer safety. *L. monocytogenes* is associated with high-risk foods like deli-meats, dairy products and seafood, with the greatest risk posed by RTE products (FAO/WHO, 2004; Chen *et al.*, 2014; Law *et al.*, 2015; Buchanan *et al.*, 2017). It should be noted, however, that there has been an increase in the number of outbreaks linked to vegetable and fruit products (Buchanan *et al.*, 2017).

Prepared-fruit is an RTE that has no listericidal treatment, like heating (Kayode *et al.*, 2020). This is because of the negative effect on the quality of the product (Parish *et al.*, 2003; James *et al.*, 2011). In some cases, use of heat sterilisation on fruit products can potentially increase the likelihood of bacterial colonisation, as seen in the case of cantaloupe (Bezanson *et al.*, 2018).The impact of climate and weather has also been shown to contribute to contamination of fruit pre-harvesting, as described by a number of authors, who elucidate the role of rainfall and temperature in their ability to impact *L. monocytogenes* spread from the environment onto fruit (Hellberg and Chu, 2016).

The incidence of *L. monocytogenes* in fruit and prepared-fruit products remains scarcely characterised as they are deemed to be “low-risk”, although incidence of outbreaks from these products have increased in the USA since 2010 (Niemira and Fan, 2012; Buchanan *et al.*, 2017; Smith *et al.*, 2019). The propensity for fruit to enable the proliferation and survival of *L. monocytogenes* is due to its moderate pH and high nutrient availability (Ukuku and Fett, 2002; Penteado and Leitão, 2004; Strydom *et al.*, 2013; Forney *et al.*, 2015; Vahidy *et al.*, 2019). Understanding the role of the

agricultural environment in its potential to cause *Listerial* contamination is beneficial to all shareholders throughout the food supply chain. Two major listeriosis outbreaks have occurred as a result of orange melons (*Cucumis melo*). The first in 2011 in the USA, where a multistate outbreak resulted in the infection of 147 people with 33 deaths and a miscarriage (CDC, 2011). A second was recorded in Australia in 2018 where 22 people were infected with seven deaths (WHO, 2018). These and other outbreaks linked to fruit (Chen *et al.*, 2016a; Buchanan *et al.*, 2017) highlight the potential for fresh and prepared-fruit to act as a vector for *L. monocytogenes*.

Monitoring of *Listeria* spp. is an effective way the food industry can identify potential practices, harbourage sites and niches throughout the supply chain from agricultural to industrial environment that enables the proliferation of *L. monocytogenes* (Orsi and Wiedmann, 2016). Furthermore, lineage typing of *L. monocytogenes* can give valuable insights into the distribution and the conditions suitable for the proliferation of different genetic clades within the species, especially those isolates deemed to be hypervirulent. This study aims to determine the prevalence of *L. monocytogenes* within the agricultural and processed prepared-fruit environment, and further assessing the distribution of lineage types.

3.3 Materials and Methods

3.3.1 Sampling method and sample processing

Isolates from the agricultural environment that were assessed in this study were obtained from sampling at cantaloupe (*Cucumis melo*) and watermelon (*Citrullus lanatus)* farms in the Eastern Cape and Limpopo provinces of South Africa (Table 3.1). Isolates from the RTE prepared-fruit processing environment were collected from the in-house *Listeria* management swabbing plan at a prepared-fruit factory located in Gauteng, South Africa (Table 3.2). All swabbing for *Listeria* was performed using sponge-sticks in 10 mL neutralising buffer (3M, USA). Sampling in the agricultural environment involved swabbing at the melon farms, within the packhouse as well as in field. A total of 84 swabs were taken between two farms in the Eastern Cape and five farms in Limpopo. A total of 240 *Listeria* isolates were analysed from both the in-house *Listeria* management programme and swabbing.

Swabs were processed according to the protocol outlined in ISO 11290:1 (2017) for the detection of *L. monocytogenes* from the food chain. Isolates were analysed according to Oxoid *Listeria* Precis method, using ONE Broth-*Listeria* and *Brilliance Listeria* Agar (Oxoid, South Africa).

Presumptive *L. monocytogenes* as well as *Listeria* spp. on *Brilliance Listeria* Agar (Oxoid, South Africa) were obtained from the prepared-fruit factory's (PFF) SANAS ISO 17025:2017 accredited

laboratory located in Gauteng and transferred to the research laboratory for further processing. Isolates obtained from swabbing at the farms were analysed by the PFF's laboratory in the Western Cape with species classification determined using the Microbact *Listeria* 12L Kit (Oxoid, South Africa). A total of 240 presumptive *Listeria* spp. were collected from the PFF's in-house *Listeria* management plan. Two colonies were then taken from each of the presumptive positive plates and grown for 24 hours in Brain Heart Infusion (BHI) broth. They were then streaked onto RAPID'L.mono™ (RLM) agar (Bio-rad, USA) in order to determine whether the isolates were *Listeria* spp. or *L. monocytogenes* – through visual identification of blue/black colonies – and to obtain pure cultures. This resulted in the recovery of 193 isolates from 101 different swabbing points.

Isolates were then prepared for storage by re-streaking onto BHI agar and culturing for 24 hours at 37^{o}C before being directly transferred from BHI agar to a sterile 25% glycerol-dH_2O in a sterile 2 ml cryotube before being stored at -20^{o}C. *L. monocytogenes* positive controls were ATCC strains 7644, 23074, and 19114.

Table 3.1 Location of swabs taken in the agricultural environment

Swabbing location	
Raw material	**Environment**
Cantaloupe in field, before and after cleaning	Crates
	Conveyor belts
	Brushes on melon cleaner
	Personnel hands
Watermelon in field, before and after cleaning	Plastic mulching
	Drains

Table 3.2 Location of swabs taken in the PFF positive for presumptive *Listeria* spp.

Swabbing location	
Raw material and final product	**Environment**
Red melon	Trolley wheels
Orange melon	Conveyor belt
Green melon	Crates
Papaya	Crate washer
Guava	Lugs
Avocado whole and pulp	Prepping knives
Banana	Peelers
Fruit salad	Chopping boards
	Drains

3.3.2 Genomic DNA isolation, PCR and RFLP analysis

3.3.2.1 PCR set-up and conditions

Glycerol stocks of isolates were streaked onto BHI agar and incubated at 37ºC for 24 hours. Isolates were then processed using the *Quick*-DNA Fungal/Bacterial Miniprep kit (Zymo Research, USA) to obtain genomic DNA.

Amplification of the 731 bp portion of the *hly* gene was analysed to confirm *L. monocytogenes.* This was performed using 0.3 mM concentration of primers *hly*F 5'-CATTAGTGGAAAGATGGAATG-3', and *hly*R, 5'-GTATCCTCCAGAGTGATCGA-3' (Blais and Phillippe, 1993). For a 25 uL mixture, the mixture contained 2 uL extracted DNA; 0.75 uL of both the forward and reverse primer (Thermo Scientific, South Africa); 9 uL of nuclease free water (BioConcept, Switzerland); and 12.5 uL of DreamTaq™ Hot Start PCR Master Mix (2X) (Thermo Fisher Scientific, Lithuania). Thermal cycling was achieved using a thermal cycler (Bio-Rad, South Africa) with the following reaction conditions: initial denaturation for 3 min at 94°C; 30 cycles of denaturation at 94°C for 40 s; annealing at 55°C for 40 s; extension at 72°C for 40 s; final extension at 72°C for 5 min.

PCR products were then separated using electrophoresis on a 1xTAE 1.2% agarose gel (Lonza, Switzerland) and visualised using EZ-Vision (Amresco, USA). Agarose gels were then viewed using

a Gel Doc™ XR+ with Image Lab™ Software (Bio-Rad, South Africa). *L. monocytogenes* ATCC 7644 was included as a positive control.

3.3.2.2 RFLP analysis

Following confirmation of *L. monocytogenes* through detection of the 731 bp *hly* amplicon, PCR products were subjected to restriction enzyme digests in order to determine the lineages of isolates as described by Rip and Gouws, (2020). The restriction enzymes used were for the classification of isolates into lineages I, II and III. A 10uL reaction contained: 2uL PCR amplicons; 1.5uL restriction enzyme; 1X final volume restriction enzyme buffer (Thermo Scientific, South Africa); 5.5uL nuclease free water (BioConcept, Switzerland). All restriction enzymes used – *Nde*I, *Hae*II and *Bsh*12851 (Thermo Scientific, South Africa) – were incubated at 37ºC for 25 min. RFLP assays were placed on ice following incubation, separated using electrophoresis on a 1xTAE 1.2% agarose gel (Lonza, Switzerland) and visualised using EZ-Vision (Amresco, USA). Agarose gels were viewed using a Gel Doc™ XR+ with Image Lab™ Software (Bio-Rad, South Africa) for bands characteristic to each of the three lineages (Table 3.3).

L. monocytogenes positive controls were included for lineages I, II and III as ATCC strains 7644, 23074, and 19114 respectively. Undigested DNA was included as a negative control.

Table 3.3 Enzymes used for RFLP analysis of *L. monocytogenes*

Serotypes	Lineage group	Enzyme	Band size (bp)
1/2b, 3b, 4b, 4d, 4e, 7	I	*Nde*I FastDigest	390, 341
1/2a, 1/2c, 3a, 3c	II	*Bfo*I FastDigest	294, 277, 160
4a, 4c	III	*Bsh*12851 FastDigest	384, 347

(Rip and Gouws, 2020)

3.4 Results and Discussion

3.4.1 Agricultural environment

Swabbing at farms in the Eastern Cape (EC) and Limpopo (L) yielded interesting findings (Table 3.4 and 3.5). The differences observed in abundance of *Listeria* spp. isolated at farms in EC in comparison to L are shown alongside temperature during sampling. Differences were observed in the relative humidity (RH), temperature and rainfall in each of the provinces during sampling (Figures 3.1-3.6). It is well established that *Listeria* spp. and *L. monocytogenes* are well adapted saprotrophs that are ubiquitous in the environment and are common in soil, water and silage (Botzler *et al.*, 1974; Weis and Seeliger, 1975; Smith *et al.*, 2018), making contamination in the prepared-fruit supply chain (PFSC) common. This also presents a challenge in determining the source of contamination. Due to the nature in which both watermelon and cantaloupe are grown – where they are in direct contact with the soil – it is imperative that they are washed or cleaned in some way prior to preparation for consumption (Ukuku *et al.*, 2012).

Table 3.4 Average temperatures and number of positive swabs from the two provinces

Region	**Average temp. (ºC)**	**Number of swabs**	**Positive *Listeria* swabs**	***L. monocytogenes***
EC	17-22	50	22	2
L	17-31.5	34	0	0

Table 3.5 Location and presumptive classification of isolates from the agricultural environment of two farms in the Eastern Cape (Farm A and B) investigated for the presence of *Listeria*

Source of swab	No. of isolates		Presumptive result by Microbact™ 12L
	Farm A	Farm B	
Watermelon	3	-	*Listeria* spp.
Cantaloupe	-	8	
Conveyor	3	1	
Sorting bin	1	2	
Personnel hands	-	2	
Soil and water	-	-	
Sorting crate	-	2	*L. monocytogenes*

Although several studies have aimed to characterise the mechanism enabling the adhesive capability of *L. monocytogenes*, specifically on cantaloupe, this remains relatively undetermined (Ukuku and Fett, 2002).

The results of sampling from Limpopo revealed an apparent absence of *Listeria* spp. and *L. monocytogenes.* This contrasted with the Eastern Cape, where *Listeria* spp. were isolated from a range of locations (Table 3.4). These findings have numerous elements to consider. *Listeria* spp. isolated from the environment, especially in high-stress environments, are influenced by σ^B stress factor expression, leaving them in a viable but nonculturable (VBNC) state (Oliver *et al.*, 2007; Smith *et al.*, 2018). Although there are numerous well-established reservoirs for *Listeria* in the natural environment like water and soil (Linke *et al.*, 2014) these numbers are low (Fenlon, 1999), with the potential for the organisms survival and proliferation within each niche remaining dependent on several factors which may increase the likelihood of contamination (Ivanek *et al.*, 2006). For instance, the survival of *Listeria* in soil and sewage can be negatively influenced by warmer, drier conditions especially for extended periods (Al-Ghazali and Al-Azawi, 1988; Ivanek *et al.*, 2006). Moisture and availability of water play a contributing role in the spread and survival of microorganisms within the soil. Linke *et al.* (2014), determined that *Listeria* spp. were more frequently isolated in soil with a lower moisture content (22.96%); they also saw the potential effect of seasonal weather as the lowest isolation rates occurred in the hottest month and the highest isolation rates in autumn, which is a seasonal occurrence noted by Weis and Seeliger, (1975) and MacGowan *et al.* (1994). In this study

we found a greater occurrence also in autumn as opposed to hotter spring months. The increased isolation of *Listeria* spp. in soil and groundwater which occurred following river flooding, supported the notion that *Listeria* dissemination is facilitated by watercourses, as well as intermittent and prolonged rainfall (Linke *et al.*, 2014; Hellberg and Chu, 2016). Furthermore, wet surfaces typically present microorganisms with a higher survivability (Davidson *et al.*, 1999; Moore and Griffith, 2002), a contributing factor to the outcomes of environmental sampling.

In this study, significantly higher temperatures during sampling in Limpopo than in the Eastern Cape were observed. Compounding the differences in temperature was the difference in amount of rainfall and chance of precipitation during and before the sampling period (Figure 3.1-3.6). Overall, these outcomes can be attributed to seasonal differences at the time of sampling. Farms in Limpopo were sampled in spring (October), whilst sampling in the Eastern Cape occurred during autumn (March). Seasonal differences in weather are supported as being significant in the isolation of *Listeria* spp. from the agricultural environment (Ivanek *et al.*, 2006; Zunabovic *et al.*, 2011; Bill *et al.*, 2014; Chersich *et al.*, 2018), as well as the role water and rainfall plays in the dissemination of the organism and the availability of microorganisms in the swabbing of wet surfaces (Jones *et al.*, 2020).

Presumptive *L. monocytogenes* isolated from sampling in the agricultural environment could not be sub-cultured from selective media. This may be due to the propensity for environmental isolates to enter a viable but nonculturable (VBNC) state. This is a survival strategy that may be brought on by a range of factors: incubation temperatures that differ from environmental norms, differences in osmotic concentrations, light exposure, limited nutrient availability and exposure to some common selective agents in enrichment media, like lithium chloride and acriflavine (Beumer *et al.*, 1996; Jacobsen, 1999; Oliver, 2010; Supanivatin *et al.*, 2012; Li *et al.*, 2014; Smith *et al.*, 2018).

The occurrence of *Listeria* spp. on the surfaces of both watermelon and cantaloupe can be linked to the way the fruit is grown, where it is in direct contact with soil. These results indicate the importance of sampling and the need for a larger sampling size, particularly in drier conditions where the organism may be under stress and occur in lower numbers than in more ambient and wet conditions. Furthermore, seasonal differences in temperature, rainfall, and relative humidity were shown to potentially increase the dissemination of *L. monocytogenes* on the surfaces of melons. Adverse weather events, like rainfall and dust storms, were found to have potentially contributed in the listeriosis outbreak that occurred due to contaminated melons in Australia in 2018 (Das, 2019). The role of contamination in the packing facility was a contributing factor in the 2011 listeriosis outbreak linked to cantaloupe in the USA (CDC, 2011; Buchanan *et al.*, 2017), as well as in the 2018 listeriosis outbreak previously mentioned in Australia (Das, 2019). By understanding the impact of weather conditions pre-harvest on *Listeria* spp. and *L. monocytogenes* dissemination, as well as the

distribution of the pathogen in the packing environment, levels of contamination in the PFF environment and the final product can be better controlled.

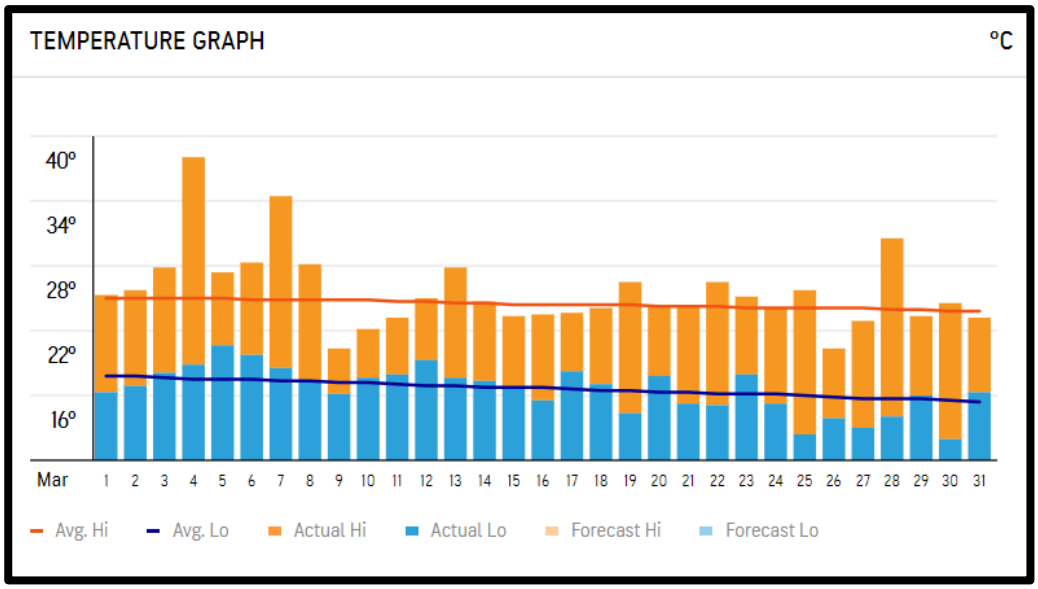

Figure 3.1 Average temperatures recorded in the area sampled in the Eastern Cape for March 2020 (Accuweather.com).

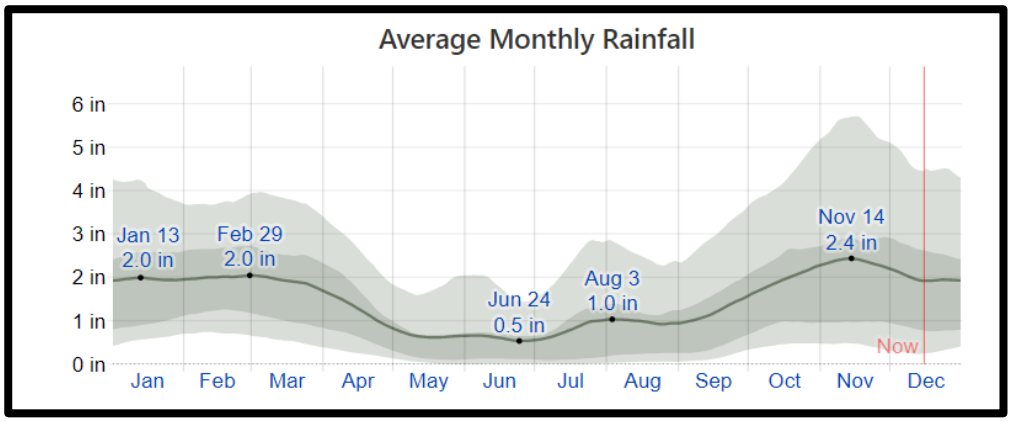

Figure 3.2 Average monthly rainfall observed in sampling in the Eastern Cape (Weatherspark.com).

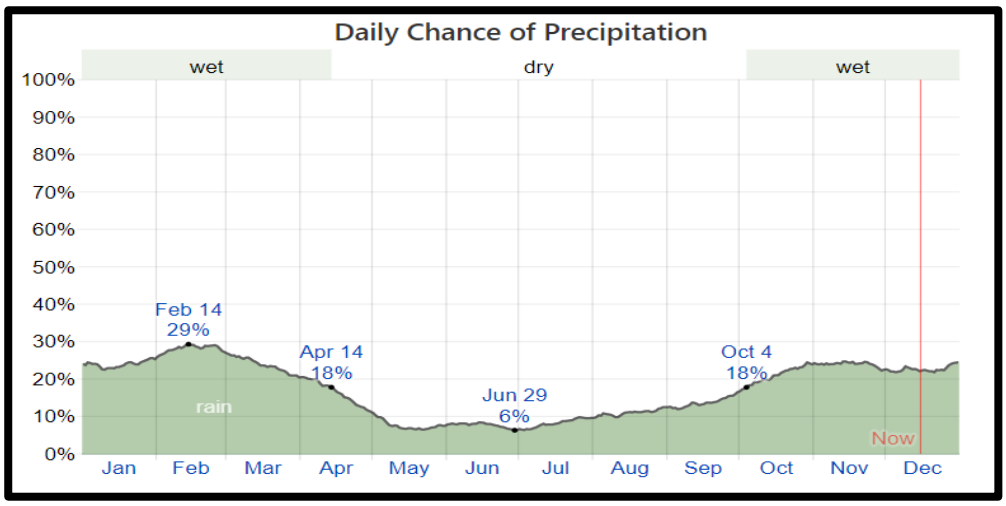

Figure 3.3 Average daily percentage chance of precipitation in sampling in the Eastern Cape (Weatherspark.com).

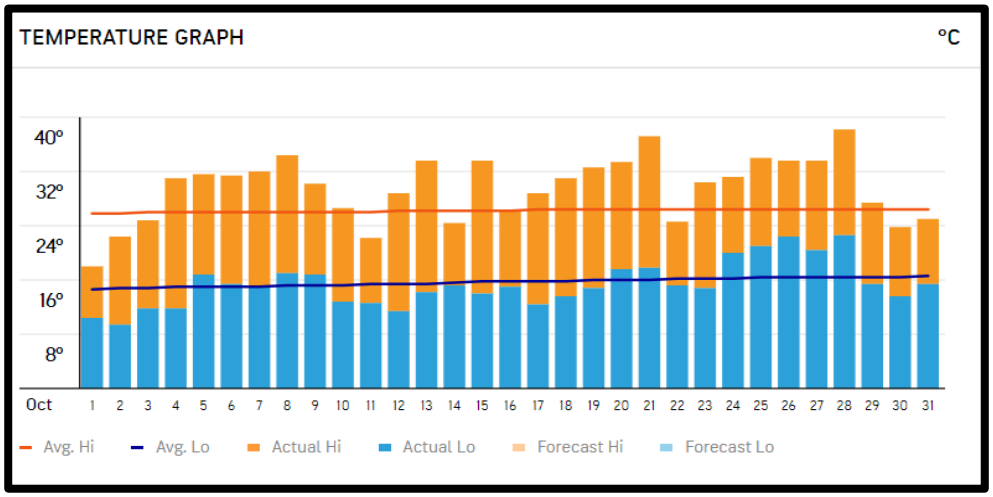

Figure 3.4 Average temperatures recorded in the area sampled in Limpopo for October 2019 (Accuweather.com).

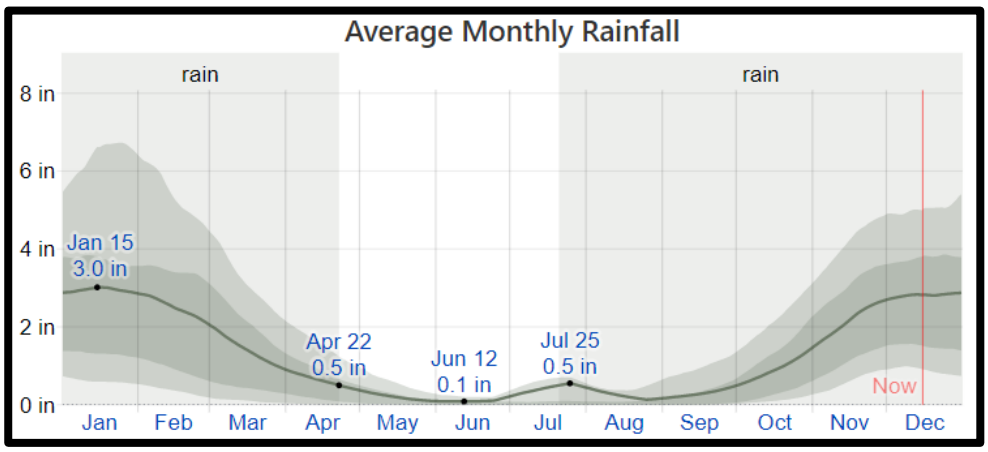

Figure 3.5 Average monthly rainfall observed in sampling in Limpopo (Weatherspark.com).

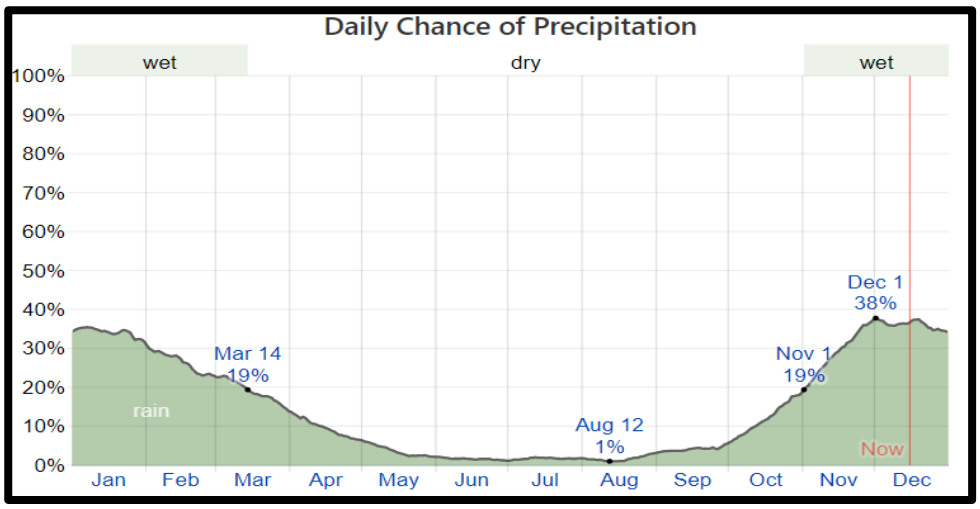

Figure 3.6 Average daily percentage chance of precipitation in sampling in Limpopo (Weatherspark.com).

3.4.2 Food processing environment

The results of the plating of isolates on RLM agar showed all 49 presumptive *L. monocytogenes* isolated from the food production environment which were confirmed through PCR amplification of the *hly*A gene, which encodes for LLO (Rodríguez-Lázaro *et al.*, 2004; Rip and Gouws, 2020). Following PCR confirmation, the RFLP analysis of isolates was used to confirm their lineage type (Table 3.6). The findings were that the majority of isolates were represented by serotypes belonging to lineage II (67%). This is typical of outcomes during other environmental sampling studies (Orsi *et al.*, 2011). Often attributed to the greater prevalence of plasmids in lineage II isolates that confer beneficial survival mechanisms, like antibiotic and sanitiser resistance, increased expression of stress factor σ^B – involved in the up-regulation of virulence genes, survival in the intestinal lumen (de las Heras *et al.*, 2011), biofilm formation and recombination rates (Orsi *et al.*, 2011).

Table 3.6 PCR-RFLP based lineage typing of isolates and their area of detection in the food processing environment. Percentages in brackets indicate the number of isolates relative to the total of each of the three sources

Source	Lineage	
	L1	**L2**
Environment	**2 (12.5%)**	**14 (87.5%)**
Chopping board	1	0
Crates	0	3
Drains	0	4
General	1	7
Raw material	**7 (20.6%)**	**17 (79.4%)**
Cantaloupe	0	1
Watermelon	1	3
Papaya	5	7
Avocado	1	5
Guava	0	1
Final Product	**7 (77.8%)**	**2 (22.2%)**
Avocado pulp	7	0
Mixed melon salad	0	2
Total	16	33
	33%	67%

The identification of *L. monocytogenes*, specifically isolates from lineage I in processed avocado, has been previously noted (Strydom *et al.*, 2013; Rip and Gouws, 2020). The occurrence of lineage I isolates in a final product is of concern as this genetic lineage is overrepresented in clinical cases and listeriosis outbreaks. The majority of listeriosis outbreaks are linked to four serovars (1/2a, 1/2b, 1/2c, and 4b) and the majority of those occurring due to the lineage I serovar, 4b (Vazquez-Boland *et al.*, 2001; Orsi *et al.*, 2011). The increased virulence in lineage I isolates is often deemed to be as a result of the fully intact *inl*A gene, encoding for internalin – highly correlated to virulence and the ability to invade cells (den Bakker *et al.*, 2010; Orsi *et al.*, 2011).

These findings (Table 3.6) corroborate those of previous researchers, in that the majority of isolates found in the food processing environment and raw material were of lineage II, and a greater proportion of lineage I isolates in final product (Sauders *et al.*, 2009; Chen *et al.*, 2010, 2020; Wu *et al.*, 2016; Henriques *et al.*, 2017; Yoshikawa *et al.*, 2018). Having said this, other findings have also indicated an overrepresentation of lineage II isolates and specifically in food products (Orsi *et al.*,

2011; Ebner *et al.*, 2015; Acciari *et al.*, 2017). Within the food processing environment, results indicated a higher prevalence of lineage II isolates (87.5%) compared to lineage I (12.5%) (Table 3.6). The occurrence of *L. monocytogenes* in drains is to be expected and has been previously noted (Gandhi and Chikindas, 2007; Strydom *et al.*, 2013; Chen *et al.*, 2017). The occurrence of isolates on chopping boards and crates may be attributed to findings that show a greater capacity of *L. monocytogenes* to form biofilms on polymer surfaces like polypropylene, polyurethane and polyvinylchloride (PVC) (Midelet and Carpentier, 2002; Poimenidou *et al.*, 2016). There is also evidence of the decreased efficacy of some common sanitisers in disinfecting and removing biofilms formed on these materials (Krysinski *et al.*, 1992; Somers and Lee Wong, 2004; Ibusquiza *et al.*, 2011; Carpentier, 2014).

The occurrence of *L. monocytogenes* in the range of whole fruit (cantaloupe, watermelon, papaya, avocado and guava) as well as in final products (avocado pulp and mixed melon salad) within the food processing facility is concerning (Ziegler *et al.*, 2018).

The occurrence of *L. monocytogenes* on both watermelon and cantaloupe has been previously studied, with the contamination of both products linked to the close proximity to the soil in which both grow (Bowen *et al.*, 2006; Kwon *et al.*, 2018). Contamination may also take place during cleaning of the fruit, as a result of internalisation through cracks, external damage or the stem scar (Macarisin *et al.*, 2017). The pulp of both of these melons also provides an adequate environment for the growth of *L. monocytogenes* due to their moderately acidic pH and high sugar and nutrient content (Ukuku and Fett, 2002; Penteado and Leitão, 2004; Fang *et al.*, 2013). Melons in particular are deemed to be a major vector for *L. monocytogenes* (Ziegler *et al.*, 2018; Marik *et al.*, 2020), with previous outbreaks of listeriosis reported in 2011 in the USA and in 2018 in Australia (McCollum *et al.*, 2013; Das, 2019). Interestingly, the isolate responsible for the outbreak in the USA in 2011 also belonged to lineage II (ST29) (Lomonaco *et al.*, 2013), whilst the outbreak in Australia was the result of a lineage I strain (ST240) (Das, 2019). The presence of *L. monocytogenes* on cantaloupe (L2) and watermelon (L1 & L2) as well as in mixed melon salad (L2) (Table 3.6), support the findings of previous authors (Ukuku *et al.*, 2012; Salazar *et al.*, 2017; Bezanson *et al.*, 2018; Huang *et al.*, 2019).

Results also indicated the potential for *L. monocytogenes* survival on the surface of papaya (L1 & L2) (Table 3.6) potentially as a result of post-harvest treatments (Raybaudi-Massilia *et al.*, 2013; Sivakumar and Wall, 2013). *L. monocytogenes* contamination of papaya is possible, with previous evidence of its isolation from the fruit (Vahidy *et al.*, 1992 as cited by Raybaudi- Massilia *et al.*, 2013). Little evidence regarding this fruit and its association with *L. monocytogenes* is available, however, it has been shown that the pulp of papaya adequately supports the growth of *L. monocytogenes*, in part due to its relatively neutral pH and available nutrients (Penteado and Leitão,

2004). This may be further exacerbated in the case of papaya which has been stored at ambient temperatures for a prolonged period of time due to the decreased acidity of the fruit pulp, which is seen as a determining factor for the survivability of *L. monocytogenes* and may have contributed to the occurrence of *L. monocytogenes* isolated from papaya in the PFF (Azene *et al.*, 2014; Ziegler *et al.*, 2018).

Avocado, as previously mentioned, has the potential to act as a vector for *L. monocytogenes* (Iturriaga *et al.*, 2002; Strydom *et al.*, 2013; Chen *et al.*, 2016b; Salazar *et al.*, 2020; Pomeroy *et al.*, 2021). Not only is the surface of avocado adequate for the attachment and survival of the organism, but internalisation through the stem scar into the pulp of intact fruit has also been observed (Chen *et al.*, 2016b). Avocado pulp typically has a neutral pH around 6.7 (Iturriaga *et al.*, 2002), making it ideal in supporting the growth of *L. monocytogenes*. The isolation of *L. monocytogenes* in this study from whole avocado (L1 & L2) (Table 3.6) may be as a result of the rough outer surface of the fruit, as with cantaloupe, enabling the attachment of bacteria and difficulty in disinfection (Wang *et al.*, 2009; Rodríguez-Garcia *et al.*, 2011). However, the occurrence of *L. monocytogenes* on whole avocado is likely as a result of contamination post-disinfection or during prepping, especially when wash water is used which increases the available water on the fruit surface better enabling *L. monocytogenes* growth and survival (Chen *et al.*, 2016b). This is not to say that pre-harvest contamination has not been identified previously, where contamination has been linked to soil and irrigation water (García-Frutos *et al.*, 2020). A greater proportion of the isolates on whole avocado were of lineage II, whilst within avocado pulp only lineage I isolates were detected (Table 3.6). Highlighting contamination from the processing environment as apposed to the fruit surface, furthermore, it may also indicate differences between the lineages ability to proliferate in avocado pulp.

The growth of *L. monocytogenes* in RTE avocado dips has been shown to be considerably more than in other RTE dips (Salazar *et al.*, 2020), with avocado pulp shown to provide a suitable medium with *L. monocytogenes* increasing 4 log CFU/g after just four hours at 22^{o}C and 1 log CFU/g after 5-8 hours at 23^{o}C (Iturriaga *et al.*, 2002; Salazar *et al.*, 2017). Results obtained in this study support the survivability of *L. monocytogenes* in avocado pulp (L1) (Table 3.6), and highlight the risk this product may play as a vector for this pathogen, especially due to the prevalence of virulent lineage I strains (100%) which are implicated in a number of clinical cases of listeriosis (Orsi *et al.*, 2011). The presence of lineage I isolates in avocado pulp is likely as a result of contamination in the production environment, as the occurrence of lineage II isolates on the surface of the fruit was far greater than lineage I, as previously mentioned. The inclusion of organic acids, typically in the form of lemon juice, in RTE avocado dips can prevent the proliferation of bacteria including *L. monocytogenes*, however, acid-stress related differences enabling greater proliferation of lineage I is unlikely, as both

lineages I and II contain the gene *lmo0038* which contributes to heat and acid stress response (Chen *et al*., 2009).

The presence of *L. monocytogenes* in RTE mixed melon (watermelon and cantaloupe) salad (L2) (Table 3.6) is anticipated, due to the survivability afforded to the pathogen by this food matrix. As stated previously, the pulp of both cantaloupe and watermelon supports the growth of *L. monocytogenes* and contamination from the rind can occur during slicing (Ukuku *et al.*, 2012; Salazar *et al.*, 2017; Bezanson *et al.*, 2018; Huang *et al.*, 2019). Proper control of storage temperature is crucial to managing the growth of *L. monocytogenes* in sliced cantaloupe and watermelon (Danyluk *et al.*, 2014), whilst preventing contamination by discarding damaged or cracked fruit is also a measure which can reduce contamination; proper disinfection and post-harvest treatment of melons is paramount to mitigating potential contamination during slicing and preparation (James *et al.*, 2011; Oliveira *et al.*, 2014; Bezanson *et al.*, 2018; Marik *et al.*, 2020). All the previously mentioned concerns support the finding that lineage II isolates were located in RTE mixed melon salad as well as on the surfaces of both fruit, suggesting contamination during prepping of the fruit as a result of a contaminated fruit surface.

Although all of the fruit products implicated have a relatively neutral pH (>4), the incidence of *L. monocytogenes* on guava (L2) (Table 3.6) is uncommon due to the fruit's high acidity (Naseer *et al.*, 2018; Ziegler *et al.*, 2018), with previous research even identifying guava extract as an effective biocide against *L. monocytogenes* (Mahfuzul Hoque *et al.*, 2007). Unfortunately, to the author's knowledge, very little research has been conducted regarding the growth and survivability of *Listeria* spp. on this fruit product, with almost no incidences of large-scale outbreaks or even recalls because of *L. monocytogenes*. However, the presence of *L. monocytogenes* on fruit is likely due to the ubiquity of the organism and the natural exposure whole fruit has to the environment.

3.5 Conclusion

Evidence of *Listeria* spp. throughout the prepared-fruit supply chain, highlights the need to ensure effective measures to manage the potential risk posed by *L. monocytogenes*. The numerous points at which contamination can occur in the agricultural environment makes source-tracking challenging, however, evidence of the role of seasonal differences in temperature, rainfall and humidity on the increased occurrence of *Listeria* spp. allows for processors and farmers to understand the need to greater mitigate risk during colder and wetter weather. Ultimately management of *Listeria* spp. in the agricultural environment can be improved by having control strategies based on seasonal changes and weather patterns. The importance of eliminating *L. monocytogenes* on raw materials at the PFF is paramount to ensuring food that is safe for the consumer, as well as eliminating the potential for the

pathogen to establish itself within the production environment. It cannot be stressed enough that *L. monocytogenes* is not exposed to any treatments in prepared-fruit products that would ensure its elimination, for this reason it is crucial that raw materials from the agricultural environment are not heavily contaminated. The distribution of lineage I and lineage II isolates within the PFF revealed a higher number of lineage II isolates within the food processing environment and on the surface of fruit. Lineage I isolates were more prevalent in final products, a significant concern given that serotypes from this lineage are typically more virulent and implicated in the majority of clinical cases of listeriosis.

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Chapter 4: Susceptibility of *L. monocytogenes* isolates from a South African prepared-fruit processing facility to sanitisers used for their management

4.1 Abstract

The risk posed by *L. monocytogenes* in RTE foods requires proper management of this ubiquitous pathogen within the food processing environment. This is especially true for RTE prepared-fruit products which are unable to be heat treated due to the negative impacts caused to the quality and sensory attributes. As a result, maintenance of the cold chain and proper disinfection and sanitizing are critical factors in reducing the growth and survival of this pathogen. Effects of sub-lethal exposure to common sanitisers like chlorine and peracetic acid, as well as genetic determinants associated with lineage types may contribute to increased resistance to sanitizing practices. The Kirby-Bauer disk diffusion method was used to determine the susceptibility of a total of 49 *L. monocytogenes* isolates collected from the prepared-fruit processing facility. A repeated measures ANOVA was used to determine the effect of source and lineage type. It was determined that Byotrol ™ quaternary-free sanitiser was more effective against *L. monocytogenes* than chlorine or peracetic acid, and that lineage type (I or II) and source (raw material, environment and final product) did not significantly affect resistance ($p > 0.05$).

4.2 Introduction

L. monocytogenes is a ubiquitous environmental saprotroph capable of surviving a range of environments and as a result, remains a major contaminant throughout the food supply chain (Gasanov *et al.*, 2005; Orsi *et al.*, 2011). This pathogen is of major concern to the food industry due to its ability to cause the foodborne disease listeriosis. While listeriosis is not as commonly reported as other foodborne pathogens, it has one of the highest mortality rates (Desvaux and Hébraud, 2006; Xayarath and Freitag, 2012). Those most at risk of contracting listeriosis are the elderly, the immuno-compromised and pregnant women (Rivero *et al.*, 2003; Bortolussi, 2008) wherein listeriosis manifests as septicaemia, meningitis, encephalitis and bacteraemia, furthermore, it can result in stillbirth and spontaneous abortion (Disson *et al.*, 2008; Camejo *et al.*, 2011).

The prepared-fruit supply chain contains a number of points at which contamination with *L. monocytogenes* can occur, in both the agricultural and industrial settings (Ukuku *et al.*, 2012;

Goodburn and Wallace, 2013; Niemira and Fan, 2012). Fruit products are not able to be heat-treated due to the negative effect high temperatures have on the quality of the final product (James *et al.*, 2011; Goodburn and Wallace, 2013; Macarisin *et al.*, 2017). This in turn creates a reliance on low temperatures and regular cleaning and disinfection of the environment, as well as of the raw material (Rico *et al.*, 2007). Although fresh produce is not typically thought of as a major vector for foodborne pathogens, there has been a drastic rise in fresh produce and fruit-related foodborne outbreaks (Chatziprodromidou *et al.*, 2018).

In order to control the pathogen *L. monocytogenes* within the industrial environment, cleaning and disinfection protocols are vital. The selective pressure created by sanitisers causes *L. monocytogenes* to adapt survival mechanisms through acquisition or mutation (Holah *et al.*, 2002; Smith *et al.*, 2008; Møretrø *et al.*, 2017b). The use of sanitisers at sub-lethal concentrations and prolonged use or exposure to one sanitiser may contribute to the selective pressure that results in the persistence of strains with sanitiser resistance (Allen *et al.*, 2016).

Some sanitiser resistance may be due to plasmids. The survival advantages associated with plasmid retention are noted in the differences in plasmid isolation between serovars from different lineages. Lineage II isolates tend to have a greater prevalence of plasmids (Orsi *et al.*, 2011) which has been hypothesised as a cause for over-representation of the lineage in environmental sampling. Previous investigations on the role plasmids play in facilitating the survival of *L. monocytogenes* in the industrial environment have elucidated the selective pressure which results from the use of common sanitisers like quaternary ammonium compounds (QACs).

Sanitiser resistance is often intrinsically related to the expression of efflux-pumps or a change in fatty acid composition (Russell, 2001; To *et al.*, 2002; Bisbiroulas *et al.*, 2011), however, it should be noted that other extrinsic factors like the state of the cells (planktonic or sessile) also plays an important role in the efficacy of sanitisers as several researchers have highlighted (Russell, 2001; Ibusquiza *et al.*, 2011). Gross debris has also been shown to interfere with sanitiser efficacy, requiring food processors to support the use of sanitisers by implementing an effective cleaning regiment (Beuchat *et al.*, 2004; Pan *et al.*, 2006).

Perhaps the most noteworthy case for assessing sanitisers in the food environment stems from the use of the quaternary ammonium compounds (QAC) like benzalkonium chloride (BC). QACs are cationic surfactants that inhibit bacteria through the breakdown of cell walls (Parish *et al.*, 2003). Poimenidou *et al.*, (2016) noted that QAC resistance amongst *L. monocytogenes* was linked to biofilm formation capacity, whilst other researchers have found that efflux pump mechanisms can be induced by exposure to BC with no differences observed in their resistance in the planktonic or sessile state (Ferreira *et al.*, 2014). Furthermore, the potential for co-selection of cadmium and BC resistance in

the *Listeria* spp. suggests the potential for the propagation of genetically-based resistance determinants to persistent strains in the industrial setting from bacteria that have survived through the agricultural environment (Mullapudi *et al.*, 2008; Katharios-Lanwermeyer *et al.*, 2012).

The selective pressure created by QACs has been noted by several researchers (To *et al.*, 2002; Mullapudi *et al.*, 2008; Møretrø *et al.*, 2017a), and has been investigated thoroughly. The potential role that QAC based resistance may have on cross-adaptation to other sanitisers is concerning for the food industry (Lundén *et al.*, 2003; Møretrø *et al.*, 2017b). The potential of decreased disinfectant susceptibility as a role of QAC resistance has fuelled the introduction of QAC-free sanitisers for the disinfection of surfaces. Byotrol ™ QFS is a regularly used QAC-free sanitiser that is becoming an integral part of ensuring adequate cleaning and food safety for some food processors.

Chlorine-based sanitisers still remain a common disinfectant in the food industry due to convenience and cost, although concern has been raised regarding the potential for harmful by-products (Parish *et al.*, 2003; Goodburn and Wallace, 2013). Chlorine inhibits bacteria through its oxidising ability, damaging cellular proteins (McDonnell and Russell, 1999). The efficacy of chlorine, applied as sodium hypochlorite, in reducing and eliminating *L. monocytogenes* on fresh produce has been described previously (Delaquis *et al.*, 2002; Beuchat *et al.*, 2004). There has, however, been evidence showing variable efficacy of chlorine-based sanitisers in eliminating *L. monocytogenes* (Folsom and Frank, 2006; Singh *et al.*, 2018). Hypochlorite has been shown to have inadequacies when used for the sanitation of produce surfaces, due to chlorine's loss of activity when in the presence of organic matter that may be present on raw materials (Parish *et al.*, 2003).

Peracetic acid (PAA) is a popular sanitiser in the food-processing industry that is effective in inactivating *L. monocytogenes* on fresh produce (Parish *et al.*, 2003; Beuchat *et al.*, 2004; Singh *et al.*, 2018). It is favoured by the food industry for its ease of use, efficacy against a wide range of pathogens, and limited by-products (Lee and Huang, 2019). Peracetic acid is also an oxidising agent, that is capable of breaking both sulfhydryl (-SH) and disulphide bonds (S-S) as well as oxidising cell proteins (Kitis, 2004). Although PAA has the potential to effectively inactivate *L. monocytogenes* on fresh produce, the minimum inhibitory concentration (MIC) has been shown to exceed recommended concentrations in some *L. monocytogenes* isolates (Poimenidou *et al.*, 2016). Peracetic acid resistance may be because of the many aforementioned factors, however assessing its effect on isolates collected from an environment where PAA is regularly employed as a disinfectant is invaluable in managing this pathogen.

To better manage the risk posed by *L. monocytogenes* it is crucial to assess the efficacy of different sanitisers commonly used within the supply chain. This study aimed to assess the efficacy of the three

sanitisers employed in the RTE processing plant to manage this pathogen using isolates obtained from a range of sources within the industrial environment.

4.3 Materials and Methods

4.3.1 Sample selection and species confirmation

Isolates used for sanitiser-susceptibly testing were acquired from the in-house *Listeria* management program at a prepared-fruit factory located in Gauteng, South Africa. Isolates were recovered from *Brilliance Listeria* Agar (Oxoid, Hampshire, England), before being re-streaked onto RAPID'L.mono™ agar (Bio-Rad, South Africa) for presumptive classification. Presumptive classification was also performed using the 3M Molecular Detection System (3M, South Africa). All presumptive *L. monocytogenes* isolates (n=49) were confirmed through PCR amplification of a 738 bp region of the *hly* gene (Blais and Phillippe, 1993); their lineages were determined through RFLP-PFGE (Rip and Gouws, 2020).

Isolates were classified by lineage type (I, II) as well as by their source of isolation (environment, raw material, and final product) (Table 4.1).

Table 4.1 Source and lineage of isolates obtained from the prepared-fruit processing facility assessed for sanitiser resistance.

Source	Lineage	
	L1	**L2**
Environment	**2 (12.5%)**	**14 (87.5%)**
Chopping board	1	0
Crates	0	3
Drains	0	4
General	1	7
Raw material	**7 (20.6%)**	**17 (79.4%)**
Cantaloupe	0	1
Watermelon	1	3
Papaya	5	7
Avocado	1	5
Guava	0	1
Final product	**7 (77.8%)**	**2 (22.2%)**
Avocado pulp	7	0
Mixed melon salad	0	2
Total	16	33
	33%	67%

4.3.2 Sanitiser susceptibility testing

The Kirby-Bauer disk diffusion test (Bauer *et al.*, 1978) was used to determine the susceptibility of *L. monocytogenes* according to the Clinical and Laboratory Standards Institute (2018) using Mueller-Hinton agar (Oxoid, South Africa) and the direct colony suspension method. Discs were placed on the Mueller-Hinton agar using callipers, after they had been soaked in sanitiser solution for 10 min. All isolates were tested in duplicate with three sanitisers selected based on their applicability in the prepared-fruit industry. The concentrations used were as specified for use in a factory environment (Table 4.2).

Table 4.2 Sanitisers assessed for efficacy against *L. monocytogenes*, their active ingredients and concentration used.

Brand name	Active ingredient(s)	Concentration used (v/v)
Ecowize™ ECO-SAN SH12	Sodium hypochlorite	2%
Ecowize™ ECO-SAN AN8	Peracetic acid	4%
Byotrol™ QFS	Dodecyl dipropylenetriamine, lactic acid and polyhexamethylene biguanide hydrochloride	1%

Isolates were classified as based on the results of the Kirby-Bauer disk diffusion test. As no standards are currently available for the expected clearance zones of these sanitisers, the isolates were categorised as either resistant or susceptible based on absence or presence of a zone of clearance.

4.3.3 Statistical analysis

Analysis of sanitiser resistance was performed using a repeated measures ANOVA for the zone diameters of each 49 isolates for the three sanitisers used, chlorine, peracetic acid and QAC-free sanitiser, determining the effect of lineage and source. This was performed using Statsistica 14.0 software (TIBCO Software, Palo Alto, CA, USA). Significant effects of lineage or source were determined by analysing least significant differences (LSD) for a 95% confidence interval (p-value $\leq$ 0.05). Significant differences of results from each effect were determined by least square means (LSM) for a 95% confidence interval (p-value $\leq$ 0.05).

Percentages for susceptibility were achieved by classifying isolates as susceptible if any zone of clearance was observed, and resistant in the absence of a zone of clearance.

4.4 Results and Discussion

The effect of sanitisers used in the industry to control this pathogen revealed variable levels of resistance. In the testing performed, the sample collection was analysed according to the area from which isolates were obtained, as well as by lineage type. Amongst the 49 isolates tested, all isolates

were susceptible to Byotrol™ QFS, varying levels of PAA resistance was noted and chlorine resistance was seen in all but four isolates (Figures 4.1 & 4.2).

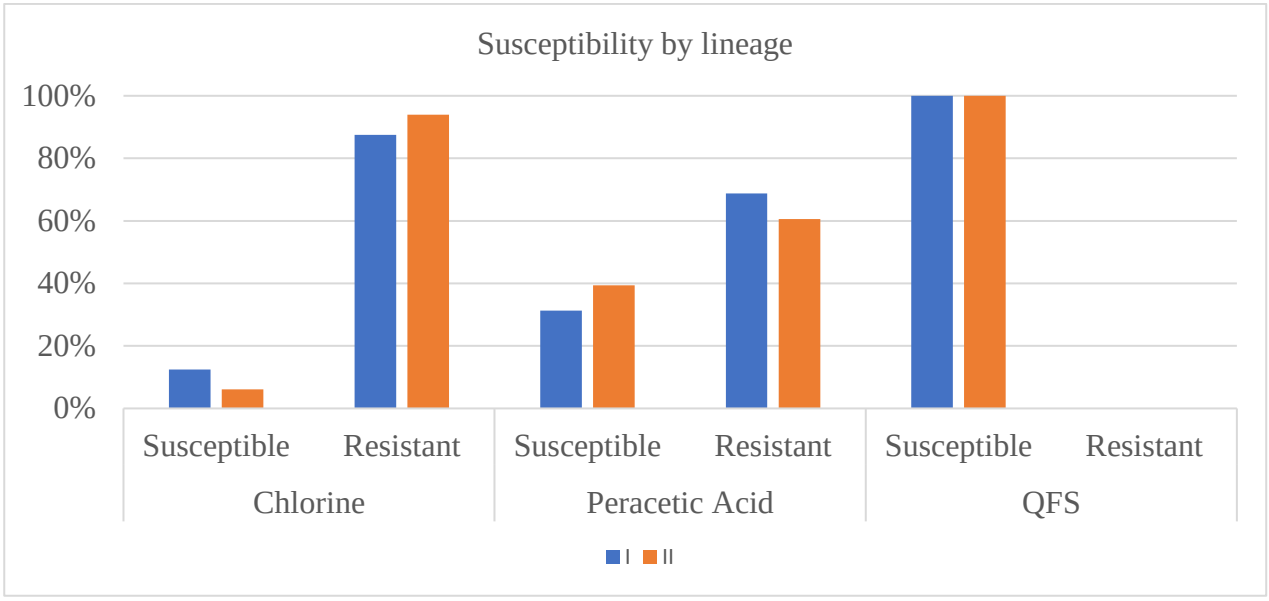

Figure 4.1 The percentages of resistance and susceptibility of *L. monocytogenes* lineages (n=49) isolated from within the industrial prepared-fruit setting.

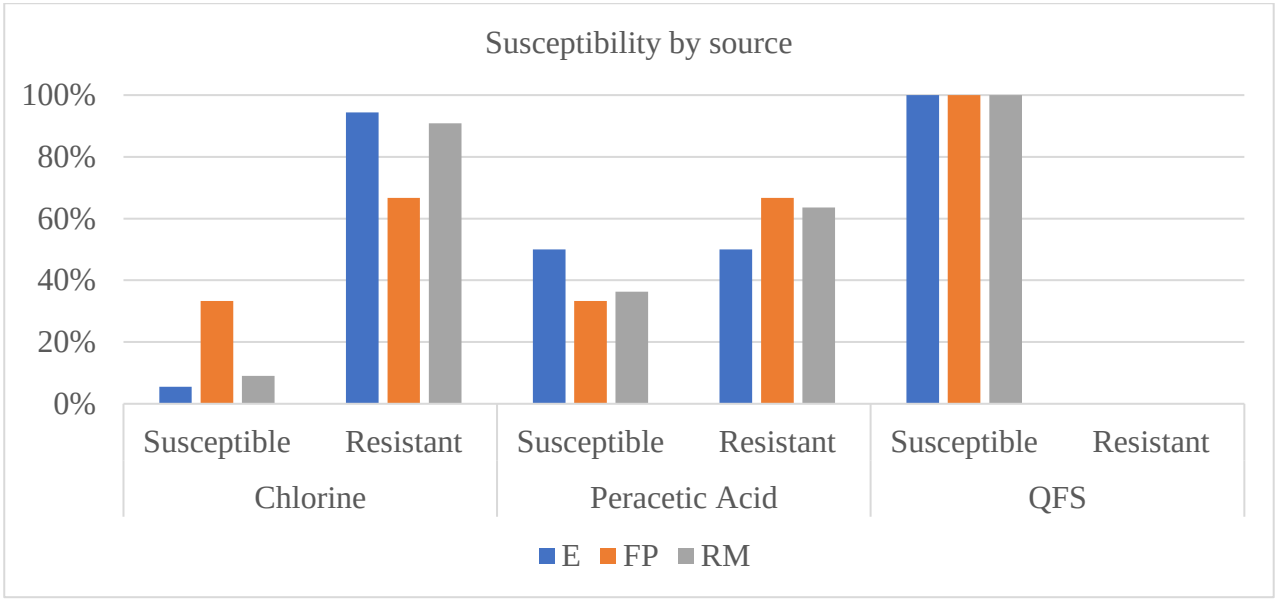

Figure 4.2 The percentages of resistance and susceptibility of *L. monocytogenes* (n=49) isolated from varying areas within the industrial prepared-fruit setting. (E: environment; FP: final product; RM: raw material).

Lineage associated resistance of *L. monocytogenes* allows an analysis accounting for the influence of genetic similarities amongst serovars (Orsi *et al.*, 2011; Carvalho *et al.*, 2019).

Of the 49 isolates (Table 4.1) tested for sanitiser resistance, 16 (32.7%) were lineage I and 33 (67.3%) were lineage II. Chlorine susceptibility was low across lineage I (12.5%) and II (6.06%) isolates, whilst a higher proportion of susceptibility to PAA was seen in lineage II isolates (39.39%) as opposed to lineage I (31.25%). Of the isolates collected both lineages showed a greater proportion of resistance to both chlorine and PAA than susceptibility (Figure 4.1 & 4.2). Evidence of resistance to both of these sanitisers amongst *L. monocytogenes* has previously been found. Folsom and Frank, (2006) expanded on adaptive chlorine resistance in *L. monocytogenes* and its link to previous sub-lethal exposure. The mechanisms that enable PAA resistance are not fully understood. Stress factor SigB and associated regulatory genes have been shown to enhance the resistance of *L. monocytogenes* to PAA (Van Der Veen and Abee, 2010). As does the efflux pump encoded by *mdr*L, which has been shown to confer QAC resistance (Gandhi and Chikindas, 2007). Our findings did not indicate that lineage II isolates that have a higher expression of SigB were any more resistant than lineage I isolates. The higher susceptibility of *L. monocytogenes* isolates to PAA is supported by several researchers (Singh *et al.*, 2018; Carvalho *et al.*, 2019; Lee and Huang, 2019) who advise its use due to its stronger oxidative properties and less harmful by-products. It should be noted that both chlorine and PAA are used on some of the raw materials at packhouses, potentially creating the opportunity to sub-lethal exposure of isolates found within the PFF.

The use of Byotrol™ QFS has not been extensively investigated in the PFSC and its application for control of *Listeria* can be considered as response to the increased occurrence of resistance to QACs, like BC, in the industrial food environment (Ortiz *et al.*, 2014; Møretrø *et al.*, 2017b). Byotrol™ QFS is a hard surface sanitiser that contains, lactic acid, dodecyl dipropylenetriamine and polyhexamethylene biguanide hydrochloride. This blend of sanitisers was highly effective against all the isolates obtained from the prepared-fruit processing environment. This may be because of its novel use or the multiple modes of action. Novel sanitisers can have a greater efficacy against organisms due to the lack of previous exposure. Dodecyl dipropylenetriamine has bactericidal effects as a surfactant (Kabara *et al.*, 1972; Drozdova, 2015), whilst polyhexamethylene biguanide hydrochloride is a bactericidal polymer that damages cell membranes and is effective against a broad range of bacteria (Zhou *et al.*, 2010; Sousa *et al.*, 2015). Lactic acid is an organic acid that has been noted to exhibit adequate antimicrobial activity and has shown efficacy against *L. monocytogenes* (Giannuzzi and Zaritzky, 1996; In *et al.*, 2013). Its use as a novel terminal sanitiser may be a contributing factor in its efficacy against *L. monocytogenes*.

The occurrence of sanitiser resistance related to the environment, final product and raw material showed similar results across all three sanitisers. Low chlorine susceptibility was noted amongst lineage I and II isolates (Figure 4.2). The highest percentage of susceptibility to PAA was seen in

isolates from the environment, and the lowest in isolates from final products (Figure 4.3). This suggests that not all *L. monocytogenes* isolates from the environment display resistance to PAA. However, continued surface disinfection and more rigorous cleaning in the high-care portion of the food processing facility may select for more resistant strains, this is of concern as the likelihood of contamination is highest during final preparation (Harris *et al.*, 2003). According to their area of isolation and for both lineages, Byotrol™ QFS proved to be the most effective sanitiser, with susceptibility shown amongst all isolates (Figure 4.2 and 4.3).

Statistical analysis indicated that there was no significant effect to sanitiser resistance as a result of the source or lineage of isolates collected from the prepared-fruit environment (p-value > 0.05) (Table 4.2 and 4.3). Although previous researchers have indicated that both lineage and source can play a role in the ability of certain strains of *L. monocytogenes* to better resist certain environmental stresses like sanitisers (Gorski *et al.*, 2008; Piercey *et al.*, 2017; Huang *et al.*, 2018), findings did not indicate that either lineage I or II had significantly different sanitiser susceptibility (p-value > 0.05). Similarly, this investigation did not find any significant difference in the sanitiser susceptibility of isolates from different environments (p-value > 0.05).

Table 4.3 Table of LS means of the zone diameter for chlorine (F (1,47) =0.194; p=0.661), PAA (F (1,47) =0.002; p=0.961) and QFS (F (1,47) =0.194; p=0.661) for lineages I and II

Lineage	N	Chlorine average zone diameter mean	Chlorine average zone diameter Std. error	Peracetic acid average zone diameter mean	Peracetic acid average zone diameter Std. error	QFS average zone diameter mean	QFS average zone diameter Std. error
1	16	0.500	0.338	2.438	0.787	16.531	1.132
2	33	0.318	0.235	2.485	0.548	16.455	1.343

Table 4.4 Table of LS means of the zone diameter for chlorine (F (2,46) =0.263; p=0.770), PAA (F (2,46) =0.067; p=0.934) and QFS (F (2,46) =1.606; p=0.212) for final product (FP), raw material (RM) and environment (E)

Source	N	Chlorine average zone diameter mean	Chlorine average zone diameter Std. error	Peracetic acid average zone diameter mean	Peracetic acid average zone diameter Std. error	QFS average zone diameter mean	QFS average zone diameter Std. error
FP	9	0.444	0.454	2.167	1.059	16.500	0.417
RM	22	0.500	0.290	2.455	0.677	16.795	0.267
E	18	0.194	0.321	2.639	0.749	16.083	0.295

Lineage II isolates of *L. monocytogenes* have been associated with a resistance to sanitisers as a result of their enhanced biofilm forming abilities – shown to better protect from disinfection in the food processing environment – and their greater propensity to harbour and transfer plasmids conferring genes beneficial to resisting common disinfectants (Borucki *et al.*, 2003; Di Bonaventura *et al.*, 2008; Orsi *et al.*, 2011; Huang *et al.*, 2018). Oxidation resistance genes were important in surviving application of sanitisers like chlorine and peracetic acid which have strong oxidative effect. Whereas resistance to sanitisers in lineage I strains have been attributed to these isolates' ability to better express genes related to oxidation robustness when in the planktonic state (*sod, fri* and *perR*) (Gorski *et al.*, 2008; Huang *et al.*, 2018).

Source-related resistance to sanitisers has been noted previously, with isolates associated with food products exhibiting a stronger oxidative stress response than those associated with the environment (Gorski *et al.*, 2008; Piercey *et al.*, 2017). *L. monocytogenes* exposure to sanitisers in certain environments throughout the food supply chain is affected by material, levels of debris and frequency of disinfection and sanitising based on risk of contamination, hence the potential development and selection of sanitiser-resistant strains related to their source of isolation (Harris *et al.*, 2003; Somers and Lee Wong, 2004; Ferreira *et al.*, 2014; Lee *et al.*, 2016). Exposure to sub-lethal chlorine concentrations in *L. monocytogenes* has been shown to increase the MIC required to eliminate the organism (Lundén *et al.*, 2003; Folsom and Frank, 2006). The converse has also been seen with Kastbjerg and Gram, (2012) finding that prolonged sub-lethal exposure did not enable resistance of isolates to exposure of certain sanitisers at higher concentrations. Furthermore, in environments that

do not undergo regular disinfection, the role of mature biofilms may greatly contribute to the sanitiser resistance of isolates within these environments (Ferreira *et al.*, 2014). Sanitiser use in the food processing environment is crucial to controlling the occurrence of *L. monocytogenes*, our findings ultimately show no significant difference with isolates from either the environment, raw materials or final product of a prepared-fruit processing facility and the susceptibility to peracetic acid, chlorine, and quaternary-free sanitiser.

A major contributor to sanitiser resistance amongst *L. monocytogenes* is improper cleaning and disinfection, this in turn leads to sub-lethal concentrations when sanitisers are applied to surfaces that are wet or covered in organic debris, as well as hard-to-reach areas that cannot be rinsed or cleaned adequately (Henriques and Fraqueza, 2017). Understanding the susceptibility of pathogens from a particular niche to substances used for their management can give insight into emerging resistance patterns amongst different lineages and isolates from different sources, allowing the effective re-evaluation of management procedures and products to better eliminate the risk of foodborne pathogens. Farms in the Eastern Cape and Limpopo used peracetic acid and chlorine in processing of melons, whilst a combination of the these two sanitizers and QFS were used at the PFF.

Furthermore, understanding the efficacy of sanitisers on different types of materials can also improve the management of *L. monocytogenes* (Lee *et al.*, 2016). The findings discussed indicate that there is an incidence of resistance to chlorine and peracetic acid in *L. monocytogenes* isolates found in the prepared processed-fruit environment, whilst resistance to a popular non-quaternary based sanitiser, Byotrol™ QFS, was absent.

4.5 Conclusion

The use of sanitisers in the prepared-fruit supply chain is paramount to ensuring that food is free from pathogens, shelf-stable and of an acceptable quality, however the increase in sanitiser resistance amongst *L. monocytogenes* is cause for the food industry to assess and ensure that cleaning protocols are effective in managing this pathogen. Ultimately, the use of chlorine at recommended concentrations is inadequate in ensuring elimination of this pathogen, however, higher concentrations bring about concerns regarding its impact on both health and the environment. Peracetic acid remains a more effective sanitiser in the control of this pathogen than chlorine, however, the occurrence of resistance to peracetic acid means that use of this sanitiser solely will not ensure complete elimination of *L. monocytogenes*. The prevalence of QAC-based sanitisers in the food industry has resulted in the development of several instances of QAC-resistant strains, prompting the use of QAC-free sanitisers, namely Byotrol™ QFS. This novel surface sanitiser showed a far greater bactericidal effect on *L.*

monocytogenes than both PAA and chlorine and should be considered for use in conjunction with these more common industrial sanitisers for the management of *L. monocytogenes* within the prepared-fruit environment. There was also no significant difference in sanitiser susceptibility due to lineages of isolates, or the source of the isolates.

These findings reflect the increased resistance of *L. monocytogenes* isolates resident in the prepared-fruit environment in South Africa to both chlorine and peracetic acid but not to Byotrol™ QFS. It should be noted that regular rotation of sanitisers used for disinfection should always be considered to hinder the development of resistance amongst persistent isolates.

4.6 References

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Chapter 5: Comparison of 3 selective chromogenic media used for the identification of *L. monocytogenes* in the South African RTE prepared-fruit supply chain

5.1 Abstract

Ready-to-eat (RTE) prepared-fruit products pose a risk as vectors for *L. monocytogenes* as these products cannot be heat sterilised due to the negative effects caused to quality and consumer appeal. For this reason, monitoring of this ubiquitous pathogen in the RTE prepared-fruit industry through the use of chromogenic agar for the detection and presumptive classification of *L. monocytogenes* in food products forms a crucial part of managing the organism. Chromogenic media has contributed to managing this pathogen and ensuring better food safety whilst also reducing the time required to achieve presumptive results. This study assessed the impact of incubation time, lineage type and source of *L. monocytogenes* isolates on the efficacy of RAPID'L.mono™, *Brilliance Listeria* Agar and Harlequin™ *Listeria* Chromogenic Agar, as well as non-selective Brain Heart Infusion agar, in their estimation of CFU/mL. Findings indicated that all the chromogenic media were comparable, and that no significant difference was observed (p-value > 0.05) as a result of lineage or source of the isolates. It was found, however, that *Staphylococcus sciuri* was capable of growth on RAPID'L.mono™ as well as *Brilliance Listeria* Agar but not Harlequin™ Chromogenic Agar. This contaminating bacterium was distinguishable but may negatively impact *L. monocytogenes* identification. Furthermore, *Enterococcus faecalis* and *Bacillus cereus* group were also capable of growth on RAPID'L.mono™ agar. The growth of these contaminating bacteria may negatively impact recovery of *L. monocytogenes* or result in false-positive results.

5.2 Introduction

The use of chromogenic media is an important tool in the detection of pathogens in food (Reissbrodt, 2004; Greenwood *et al.*, 2005; Angelidis *et al.*, 2015). There are several types chromogenic media available for use in presumptive identification of *Listeria*. *L. monocytogenes* is a pathogen that is regularly tested for in the food industry; it is a robust organism capable of surviving temperatures as low as 0^{o} C, salt concentrations of 10% (Ribeiro *et al.*, 2006) and a pH range of 4.7-9.2 (Zunabovic *et al.*, 2011). Its ability to cause listeriosis in immunocompromised individuals makes *L. monocytogenes* a serious contaminant. Listeriosis is a life-threatening infection caused by *L.*

monocytogenes, although cases of listeriosis caused by *L. innocua* and *L. seeligeri* have been noted (Rocourt *et al.*, 1986; Favaro *et al.*, 2014). *L. monocytogenes* is of concern due to the high mortality caused by listeriosis (Allen *et al.*, 2016). The environment and products of food processors are regularly tested to check for the presence of this pathogen, as well as other *Listeria* spp. which can indicate environments with the potential for *L. monocytogenes* contamination (Orsi and Wiedmann, 2016). Although fresh and processed fruit and vegetables are not deemed to be major vectors for *L. monocytogenes*, occurrence of outbreaks linked to these food products, as well as the growing consumer interest is cause for adequate monitoring in the industrial environment and in the final product (Warriner *et al.*, 2009; Hoelzer *et al.*, 2012; Chatziprodromidou *et al.*, 2018).

Testing for *L. monocytogenes* can be achieved through various techniques from the highly-discriminative genotypic methods like PCR confirmation, RFLP lineage typing and WGS (Bubert *et al.*, 1999; Smith *et al.*, 2019; Rip and Gouws, 2020) to testing of biochemical reactions that can allow differentiation between species, like the CAMP test (Christie *et al.*, 1944; Allerberger, 2003).

Chromogenic media allows the distinction of *Listeria* spp. and *L. monocytogenes* through the use of selective agents that inhibit the growth of competing bacteria. Differentiation is achieved through the incorporation of compounds that enable colour or colony type identification, utilising specific enzymatic functions associated with *L. monocytogenes* and other *Listeria* spp. (Ottaviani *et al.*, 1997; Jacobsen, 1999; Beumer and Curtis, 2003). The requirement for the media to be selective is paramount to eliminating contaminating bacteria that may result in false positives and the masking of *L. monocytogenes*. The propensity for *L. monocytogenes* to reside in biofilms, enhances the chance of isolating bacteria other than *Listeria* during sampling (Rieu *et al.*, 2008; Di Ciccio *et al.*, 2012; Zilelidou *et al.*, 2016).

Evidence suggesting that microorganisms other than *Listeria* can be cultured on selective chromogenic media is not totally uncommon, as incidences of *Bacillus* spp. and *Enterococcus* spp. and *Staphylococcus* spp. isolation has been noted previously (Park *et al.*, 2014; Angelidis *et al.*, 2015). However, there is a fine balance between inhibition of non-*Listeria* isolates and the impact of selective agents on the enumerability of certain lineages of *L. monocytogenes*, which remains contentious (Beumer *et al.*, 1996; Jacobsen, 1999, Bruhn *et al.*, 2005).

The differentiation of *Listeria* spp. from other bacteria on chromogenic agar can be achieved through a number of biochemical reactions specific to the organism, with the most utilised being the species ability to hydrolyse esculin (McLauchlin, 1987; Fraser and Sperber, 1988). Presumptive identification of *L. monocytogenes* can be achieved through the use of the CAMP test, whereby the haemolytic ability of *L. monocytogenes* is expressed on blood agar through the synergism created by the presence of *Rhodococcus equi* and *Staphylococcus aureus* (Christie *et al.*, 1944; McKellar, 1994). This method

can be used in conjunction with Gram staining and other biochemical indicators, most commonly the inability to ferment xylose – a distinguishing characteristic enabling differentiation between *L. ivanovii* and *L. monocytogenes* (Allerberger, 2003). Phenotypic differentiation remains a presumptive means of identification for *L. monocytogenes* as genotypic anomalies can never be discounted (Gracieux *et al.*, 2003).

Identification of *L. monocytogenes* on chromogenic selective agar is achieved through mechanisms that utilise enzymatic reactions specific to *L. monocytogenes*, namely phosphatidylinositol-specific phospholipase C (PI-PLC) and broad-range phospholipase C (PC-PLC) (Smith *et al.*, 1995). Both enzymes play a crucial role in the virulence of *L. monocytogenes* and therefore make their use in selective media more specific to *L. monocytogenes* than that of haemolysis, which is also present in *L. ivanovii* and has been noted in non-pathogenic *L. innocua* and *L. seeligeri* (Rocourt *et al.*, 1986; McKellar, 1994; Favaro *et al.*, 2014).

Today's culturing methods are made specific and effective against determination of *Listeria* species because of the advent of chromogenic agar. The first effective chromogenic agar available commercially was ALOA (Agar *Listeria* according to Ottaviani and Agosti) (Ottaviani *et al.*, 1997), which could distinguish between pathogenic and non-pathogenic *Listeria* spp. due to the activity of phosphatidylinositol-specific phospholipase C (PI-PLC) and to a lesser extent phosphatidylcholine phospholipase (PC-PLC). Chromogenic media are separated into two categories with the first utilising cleavage by PI-PLC of L-α-phosphatidylinositol (Willis *et al.*, 2006; Park *et al.*, 2014). This results in a white ring of precipitation zone around the pathogenic colony, combined with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside for detection of β-D-glucosidase, which occurs in all *Listeria* spp. resulting in turquoise colonies (Restaino *et al.*, 1999; Reissbrodt, 2004). Media that make use of these chemical reactions include ALOA and *Brilliance Listeria* Agar (BLA) (Formerly Oxoid Chromogenic *Listeria* Agar (OCLA)) as well as Harlequin™ (Beumer and Curtis, 2003; Reissbrodt, 2004; Hegde *et al.*, 2007).

The second group of media includes RAPID'L.mono™ Medium (Bio-Rad) which utilises 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate for the identification of pathogenic *Listeria* colonies which appear as a blue-turquoise colour, whilst non-pathogenic colonies appear white in colour. *L. monocytogenes* is further differentiated from *L. ivanovii* by the lack of xylose fermentation, resulting in no change in the red colour of the agar (Reissbrodt, 2004).

The potential role of lineage type on a *L. monocytogenes* isolates ability to be cultured on selective media is perhaps most well-known with regards to the ability of lineage II isolates to outcompete lineage I isolates in University of Vermont Medium (UVM) (Bruhn *et al.*, 2005). This has been postulated to be as a result of the influence of selective agents within the media or potentially the

virulence of isolates, and has been thought to be the cause of the overrepresentation of lineage II isolates in environmental monitoring (Gracieux *et al.*, 2003; Bruhn *et al.*, 2005).

The efficacy of chromogenic media is multi-faceted. The ability to select for *Listeria* and then distinguish the pathogenicity is fundamental. Furthermore, the need to effectively select for the bacterium of interest without creating intra-species selective pressure is necessary for the monitoring and analysis of persistent *Listeria* communities in the industrial environment. The aim of this research chapter was to compare the ability of different chromogenic agars to select and enumerate *L. monocytogenes* and select against contaminating bacteria.

5.3 Materials and Methods

This research project employed the use of pure glycerol stocks of 20 *L. monocytogenes* and four *S. sciuri* isolated from the in-house *Listeria* management plan of a prepared-fruit factory. Isolates were plated on three selective media and one non-selective media, as well as plating of samples from two cantaloupe inoculated with a bacterial mixture. This was done in order to determine the effect of medium substrate and inherent microflora on detection and enumerability of the three chromogenic media.

5.3.1 Plating of pure cultures

5.3.1.1 Sample selection and species confirmation

Isolates used for plating on agar were obtained from the in-house *Listeria* management program at a prepared-fruit factory (PFF) located in Gauteng, South Africa. Isolates were recovered from *Brilliance Listeria* Agar (Oxoid, Hampshire, England), before being re-streaked onto RAPID'L.mono™ agar (Bio-Rad, South Africa) for presumptive classification. All presumptive *L. monocytogenes* isolates (n=49) were confirmed through PCR amplification of a 738 bp region of the *hly* gene (Blais and Phillippe, 1993); their lineages were determined through RFLP-pulsed field gel electrophoresis (PFGE) (Rip and Gouws, 2020). Along with pure isolates of *L. monocytogenes*, four *Staphylococcus sciuri* which were isolated in conjunction with *L. monocytogenes* from swabs taken in the environment were also included. As stated, these isolates were found to be natural contaminants during swabbing for *L. monocytogenes* in the PFF. These were confirmed by Vitek MS – an automated mass spectrometry microbial identification system – courtesy of Mérieux NutriSciences (Cape Town, South Africa). A total of 25 isolates were selected for analysis on chromogenic agar

(Table 5.1 and 5.2) along with five combinations of *S. sciuri* and *L. monocytogenes* or *L. innocua* and *L. monocytogenes*.

Table 5.1 Isolates chosen from the prepared-fruit processing facility for plating on chromogenic agar

Source	Species	Lineage	Number of isolates
Final product	*L. monocytogenes*	I	2
		II	0
Environment	*L. monocytogenes*	I	1
		II	8
Raw material	*L. monocytogenes*	I	1
		II	5
	S. sciuri	-	4
American Type Culture Collection	*L. monocytogenes*	I	1
		II	1
		III	1
	L. innocua	-	1

Table 5.2 Samples plated on chromogenic agar

Sample plated	Number of samples
L. monocytogenes	20
L. innocua	1
S. sciuri	4
S. sciuri and *L. monocytogenes*	4
L. innocua and *L. monocytogenes*	1

5.3.1.2 Sample preparation

Twenty-five (Table 5.2) isolates were grown from glycerol stocks in 10 mL of buffered peptone water for 48h at 37^{o} C. Test tubes containing the inoculates were then stored for no more than three days at 4^{o} C. This was done to stop growth before serial dilutions were prepared.

5.3.1.3 Plating

Serial dilutions of the 25 isolates, incubated for 48 hours in buffered peptone water (BPW), were prepared in a saline solution according to Sanders (2012) before 0.1 mL of 10^{-6}, 10^{-7} and 10^{-8} were spread plated onto RAPID'L.mono™ (RLM) (Bio-Rad, South Africa), *Brilliance Listeria* Agar (BLA) (Oxoid, South Africa), Harlequin™ *Listeria* Chromogenic Agar (HAR) (Neogen, South Africa) and non-selective Brain Heart Infusion Agar (BHI) (Oxoid, South Africa) in duplicate. Five sets of two different isolate dilutions were both plated onto the media as a mixture as previously mentioned (Table 5.2). Plates were then incubated at 37^{o}C for 24h and 48h as stipulated in ISO 11290:1-2017. Plates were counted manually and with a Protos 3 colony counter (Synbiosis, England).

Enterococcus faecalis LMG 13566 was used as a negative control, while *L. monocytogenes* ATCC 7466, *L. monocytogenes* ATCC 19114, *L. monocytogenes* ATCC 23074 and *L. innocua* ATCC 33091 were used as positive controls. In the instances where more than one isolate was plated onto an agar, half the plating volume from each sample dilution was pipetted onto the agar before being spread plated.

5.3.1.4 Statistical analysis

Analysis of the plating of *L. monocytogenes* isolates on three selective chromogenic agar and one non-selective agar was conducted using a repeated measures ANOVA. Findings indicated the interaction of agar type, lineage, species and source on the enumerability of *L. monocytogenes* on the four agars.

Colony forming units (CFU) that were recorded on the four agar plates over the three dilutions were converted to CFU/mL for dilutions with colony counts on agar between 30 and 300. A repeated measures ANOVA was performed for the four different agars CFU/mL, assessing the effect of species (*S. sciuri*, *L. monocytogenes* and a mixture of the two), lineage type of *L. monocytogenes* (I and II) and source of *L. monocytogenes* from the prepared-fruit environment (environment (E), raw material (RM), final product (FP) and American Type Culture Collection (ATCC).

Significant effects were determined by a p-value ≤ 0.05 for a 95% confidence interval. Significant results in each group were identified by LSM means (p-value ≤ 0.05) using a 95% confidence interval.

5.3.2 Spiking of cantaloupe samples

Store-bought cantaloupes were used to determine the influence of substrate matrix and natural microbiota on the enumerability of *L. monocytogenes* on selective chromogenic agar. Two cantaloupes were acquired from a major retailer, three 25g samples of each cantaloupe (including rind) were then inoculated with two different mixtures containing three bacterial isolates (Table 5.3) as well as one control sample that was not inoculated. Spiking of isolates was done according to spiking guidelines for validation of the 3M™ Molecular Detection System (3M, South Africa), with 20 cells from each of the three isolates used for each inoculated sample. This was calculated using the CFU/mL recorded on the non-selective BHI agar. These were then homogenised in half-Fraser broth (Oxoid, South Africa) by stomaching for one minute (Interscience, South Africa) before plating according to ISO 11290-1:2017. Non-*Listeria* isolates obtained from whole cantaloupe were classified using Vitek-MS.

5.3.2.1 Sample selection and species confirmation

Results of the enumerability of the 25 isolates used in the assessment of selective chromogenic agar were assessed for their use in the spiking of cantaloupes. The CFU/mL of isolates on non-selective BHI agar (Oxoid, South Africa) was analysed to determine appropriate volumes of dilutions to be used in the spiking of cantaloupe samples with strain mixtures containing three isolates (Table 5.3).

Table 5.3 Samples and strain mixtures used for inoculation on store-bought cantaloupe

Sample	Strain mixture used for inoculation
1&2A	*L. monocytogenes* ATCC 7644; *L. monocytogenes* ATCC 19114; *L. innocua* ATCC 33091
1&2B	*L. monocytogenes* lineage I isolated from watermelon; *L. monocytogenes* lineage II isolated from cantaloupe; *S. sciuri* isolated from watermelon
1&2C	None

5.3.2.2 Plating

Plating of inoculated cantaloupe was conducted identically to that of the pure glycerol stocks, however, in order to determine the role of natural microflora present on the cantaloupe, non-selective plate count agar (Oxoid, South Africa) was also included.

5.4 Results and Discussion

5.4.1 Comparison of chromogenic media

The effect of the species (L – *L. monocytogenes*, S – *S. sciuri*, M – Mixed) plated on the four agars over 24 and 48 hours showed a significant effect (p-value ≤ 0.05) for RLM and HAR (Figure 5.1, 5.2 & 5.3), with a significant difference (p-value ≤ 0.05) observed for the least square means (LSM) of *L. monocytogenes*, with higher CFU/mL observed after 48 hours. Species, however, did not exhibit a significant effect on CFU/mL for BLA (p-value > 0.05) (Figure 5.4), although a significant difference (p-value ≤ 0.05) was observed with *L. monocytogenes* over 24 and 48 hours, with higher LSM seen at 48 hours.

Non-selective BHI also showed a significant effect (p-value ≤ 0.05) on CFU/mL as a result of species, indicating a significant difference (p-value ≤ 0.05) in the mixture of *S. sciuri* and *L. monocytogenes* with lower CFU/mL noted after 48 hours (Figure 5.5), potentially due to the overlapping effect of *S. sciuri* colonies.

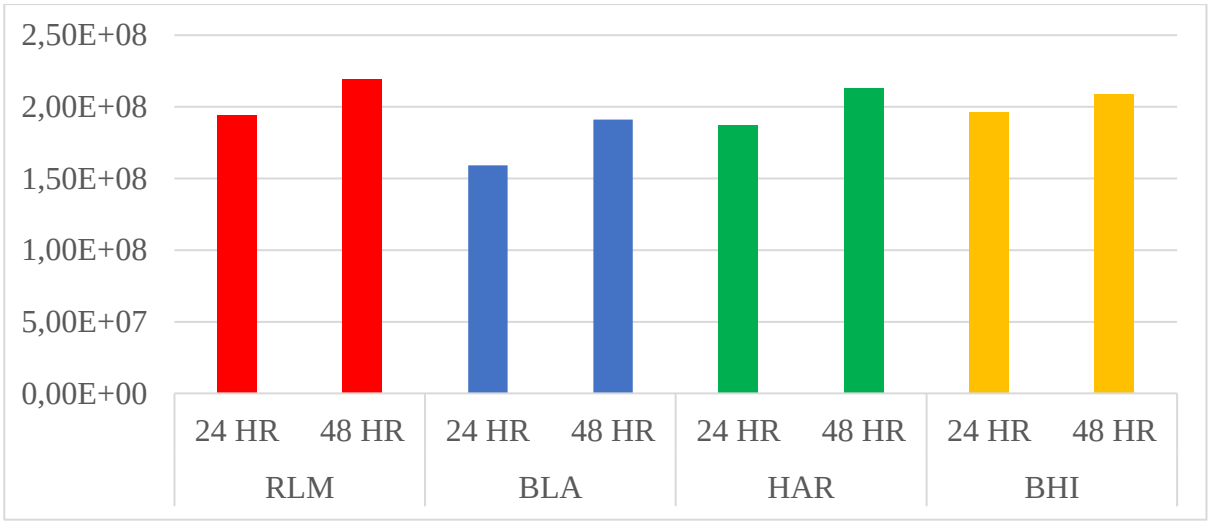


Figure 5.1 Average CFU/mL for all *L. monocytogenes* plated on the four agars over 24 and 48 hours**.**

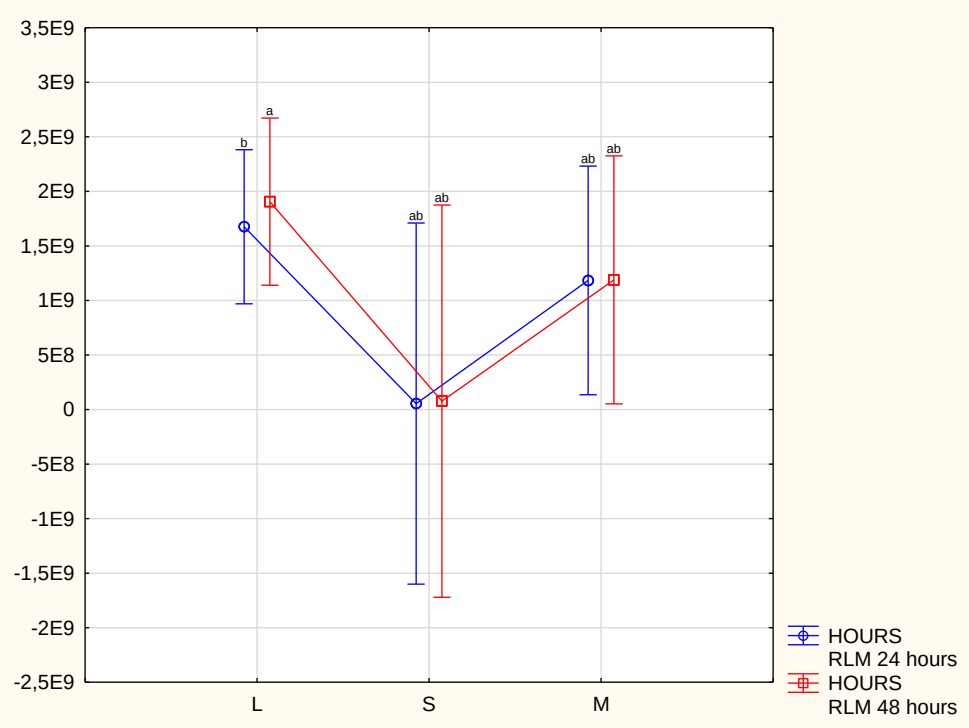


Figure 5.2 LSM of CFU/mL for lineages plated onto RLM for 24 and 48 hours. F (2,33)=3.5060, p= 0.04162. Vertical bars denote 0.95 confidence intervals.

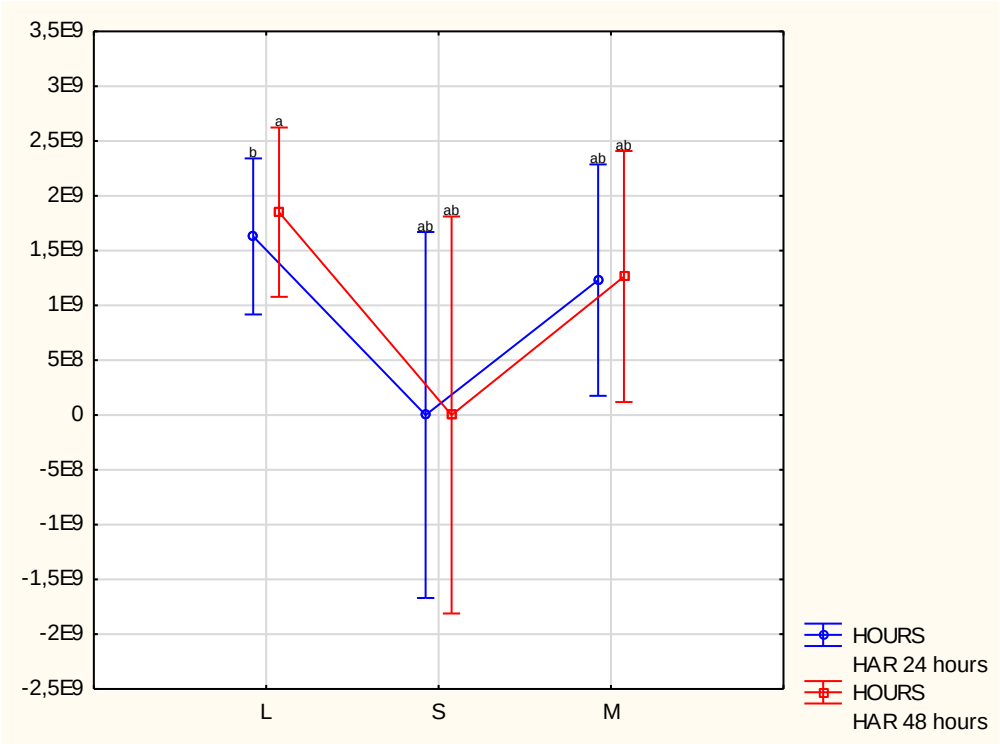


Figure 5.3 LSM of CFU/mL for species plated onto HAR for 24 and 48 hours. F (2,33)=3.5120, p= 0.04142. Vertical bars denote 0.95 confidence intervals.

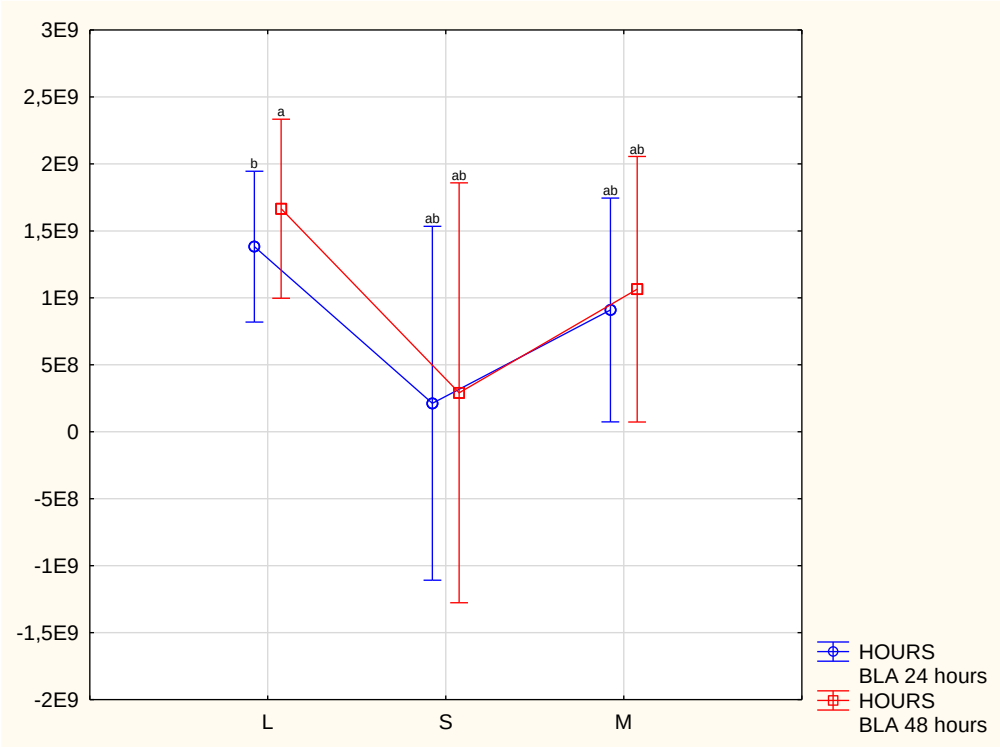


Figure 5.4 LSM of CFU/mL for species plated onto BLA for 24 and 48 hours. F (9,45)=1.0308, p= 0.3919. Vertical bars denote 0.95 confidence intervals.

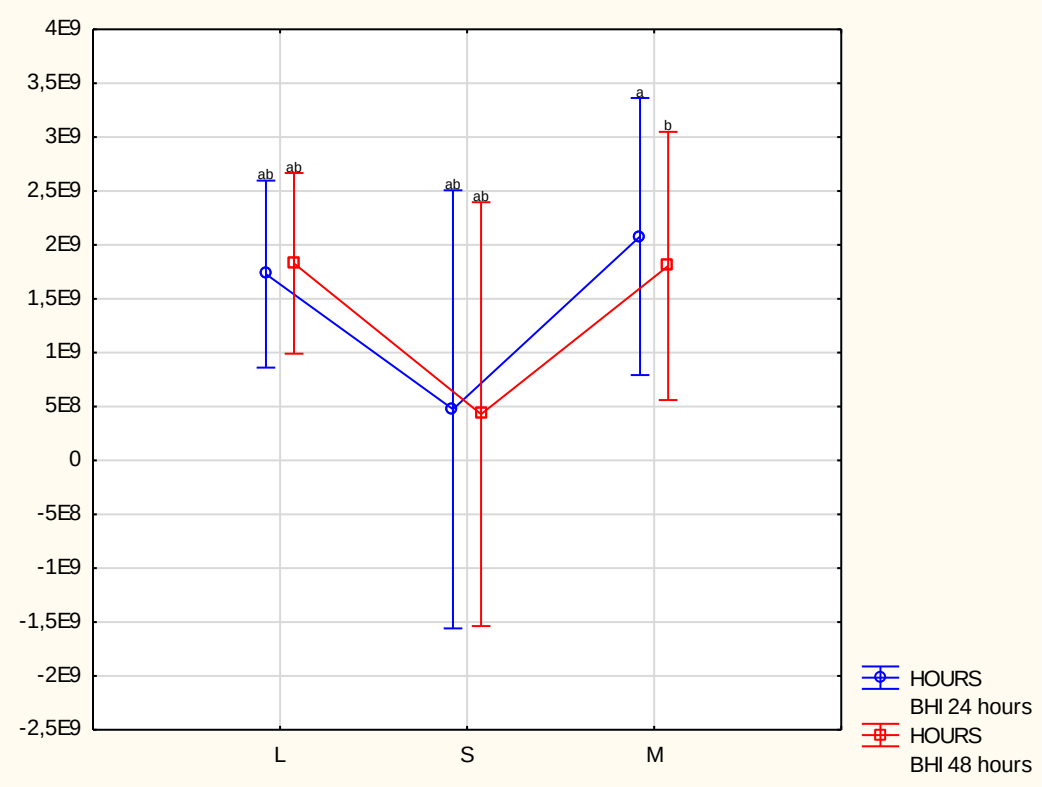


Figure 5.5 LSM of CFU/mL for species plated onto BHI for 24 and 48 hours. F (2,33)=7.6915, p=0.00181. Vertical bars denote 0.95 confidence intervals.

Results from the repeated measures ANOVA for lineages I and II indicated that lineage type had no significant effect on CFU/mL on each of the four agars (Figure 5.6). Lineage II isolates CFU/mL on BLA, which utilises the PC-PLC for detection, differed significantly (p-value ≤ 0.05) from CFU/mL of the same isolates on RLM, and BHI (non-selective) in that the LSM was lower. This highlighted the difference in recovery of lineage II isolates on media that utilises PI-PLC (RLM), which indicated higher CFU/mL as opposed to media utilising PC-PLC (BLA), although no difference was observed between HAR which also utilises PC-PLC for detection, and RLM or BLA.

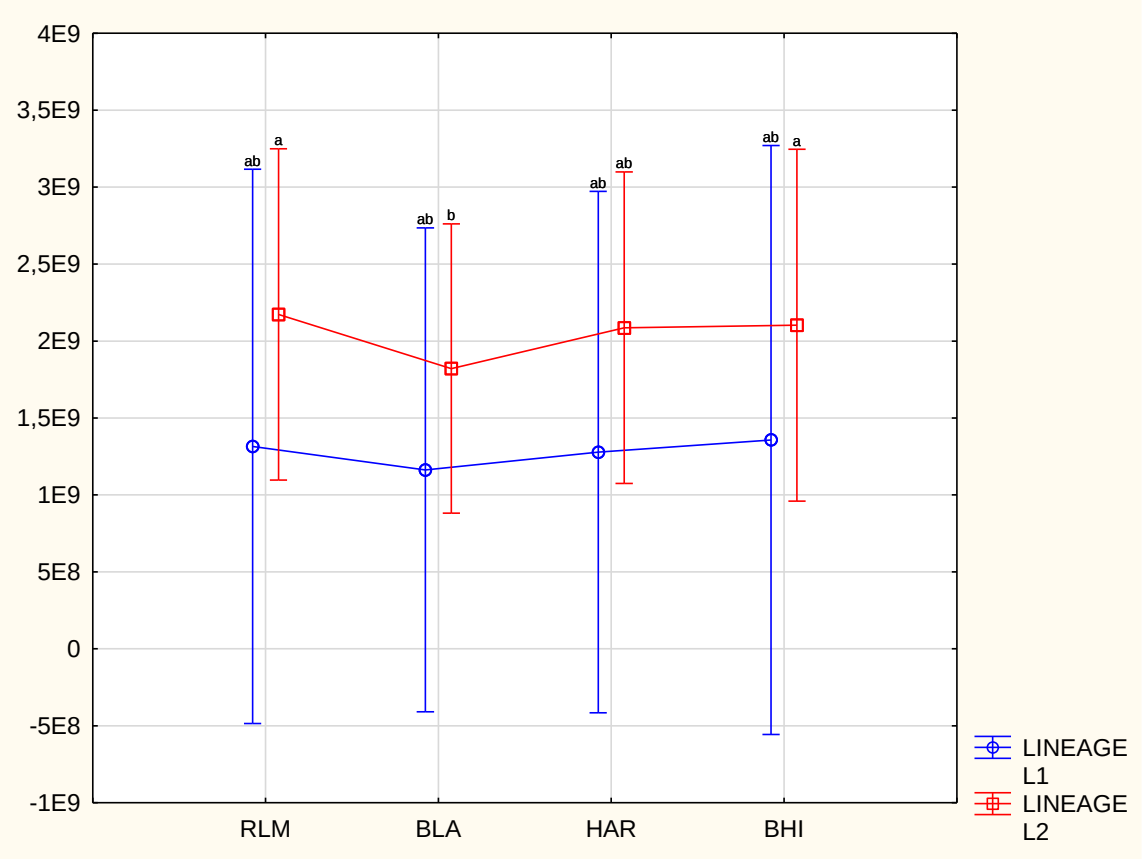


Figure 5.6 LSM for lineages plated on four different agars. F (3,51)=2.0083, p= 0.89534. Vertical bars denote 0.95 confidence intervals.

Similarly, source was also found to have no significant effect on CFU/mL for the four agars. The source of isolates may influence their resistance to selective agents. CFU/mL for isolates from the environment plated on BLA differed significantly (p-value ≤ 0.05) from environmental isolates plated on the four other agars showing a lower LSM (Figure 5.7). It should be noted, however, that there was no significant difference (p-value > 0.05) in CFU/mL of environmental isolates between BLA and HAR at 48 hours (data not shown).

The occurrence of non-*Listeria* growth on RLM and BLA (Figure 5.9) was also noted. *S. sciuri* was capable of growth on both RLM and BLA, whilst *Enterococcus faecalis* and *Bacillus cereus* group was also capable of growth on RLM.

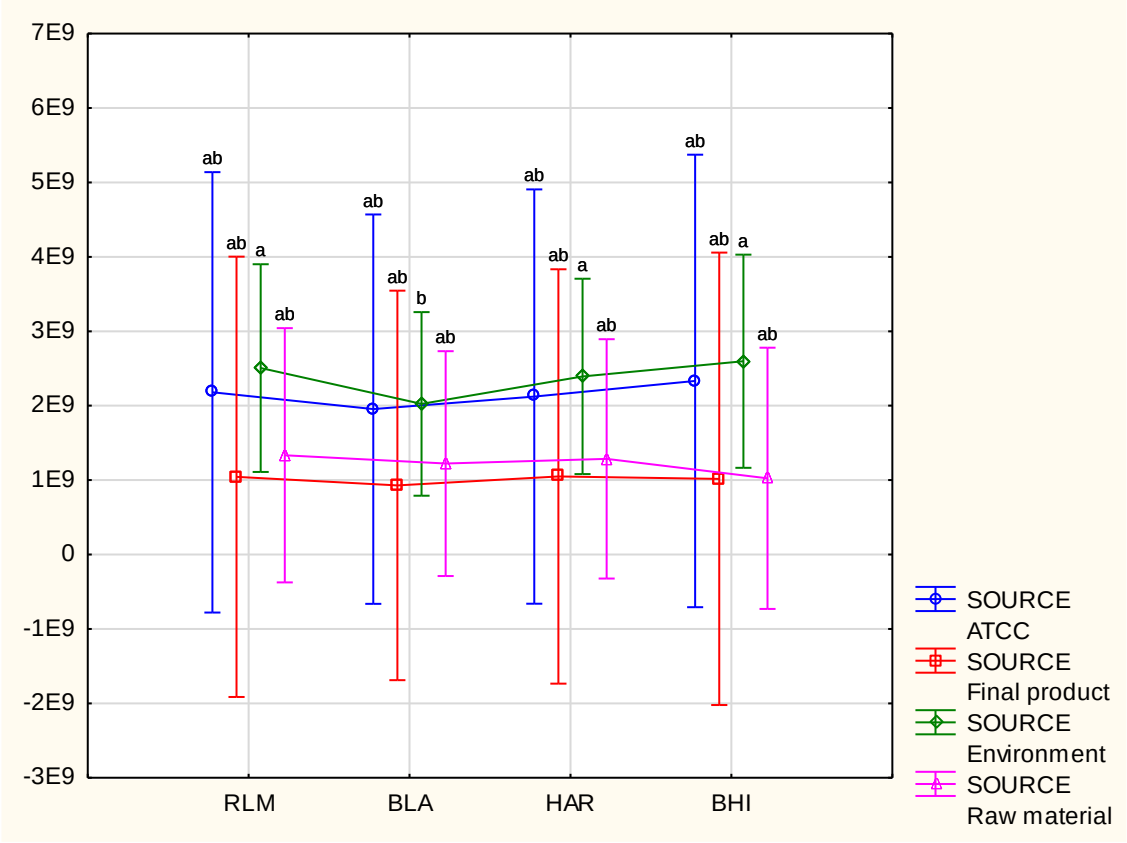


Figure 5.7 LSM for isolates from different sources plated on to four different agars. F (9,45)=1.0308, p= 0.43101. Vertical bars denote 0.95 confidence intervals.

Chromogenic media used in the detection of *L. monocytogenes* identifies the organism based on enzymatic reactions specific to the organism. Some of the earliest selective media like PALCAM and Oxford agar rely on the detection of *Listeria* spp. through esculinase activity, but are unable to differentiate between *Listeria* spp. and pathogenic *L. monocytogenes* and *L. ivanovii* (Hegde *et al.*, 2007). Most modern chromogenic media identify *Listeria* spp. based on the cleavage of 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside by β-D-glucosidase, while L-α-phosphatidylinositol or soya lecithin is included for the detection of the phosphatidylcholine-specific phospholipase C (PC-PLC) or lecithinase enzymatic system. An alternative method for identification of *L. monocytogenes* is to exploit the function of phosphatidylinositol-specific phospholipase C (PI-PLC) (Figure 5.8). Phospholipases are virulence factors which enable *L. monocytogenes* to breakdown phospholipids associated with cell membranes and vacuoles (Smith *et al.*, 1995).

In this study, both HAR and BLA utilised the cleavage of a chromogenic substrate X-glucoside by β-D-glucosidase, present in all *Listeria* spp., to presumptively identify *Listeria* spp. through the formation of a blue/green colony, which is further differentiated as either *L. monocytogenes* or *L. ivanovii* by the formation of an opaque halo as a result of PC-PLC hydrolysis of soya lecithin or phosphatidylinositol (Ottaviani *et al.*, 1997; Vlaemynck *et al.*, 2000; Reissbrodt, 2004; Angelidis *et*

al., 2015). BLA contains lithium chloride (inhibition of *Bacillus* spp.), polymyxin, ceftazidime and nalidixic acid (inhibition of Gram-positive and Gram-negative bacteria) and amphotericin B (inhibition of yeasts) as selective agents (Vlaemynck *et al.*, 2000) and soya lecithin as a differential supplement, whilst HAR contains lithium chloride, ceftazidime, polymyxin, nalidixic acid and cycloheximide (inhibition of yeasts) (Vlaemynck *et al.*, 2000) and phosphatidylinositol as a differential supplement.

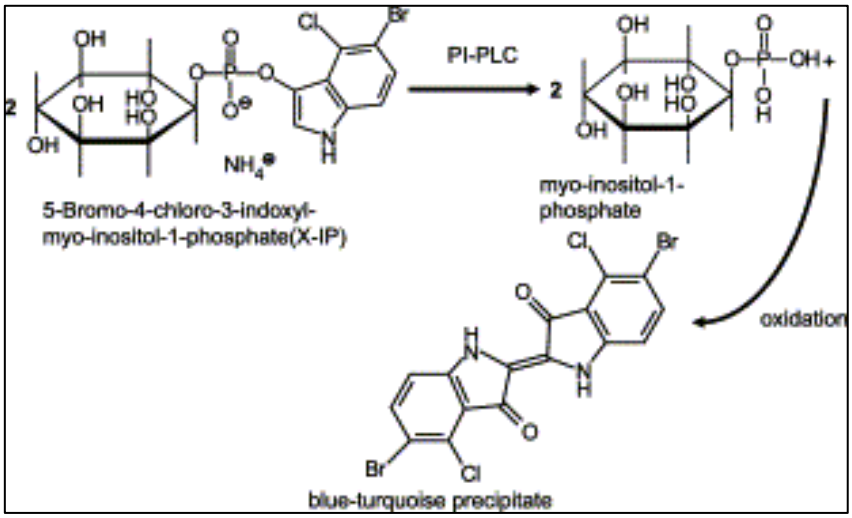


Figure 5.8 Detection of phosphatidylinositol-phospholipase C (PI-PLC) by use of X-IP (Reissbrodt, 2004).

RLM differs from BLA and HAR in that *L. monocytogenes* and *L. ivanovii* isolates are distinguished from other *Listeria* spp. by the hydrolysis of X-inositol phosphate by PI-PLC, which in turn results in colonies appearing blue whilst non-pathogenic *Listeria* spp. appear white. *L. monocytogenes* are then further differentiated from *L. ivanovii* by their inability to ferment xylose which results in the agar remaining a red colour whilst *L. ivanovii* fermentation of xylose causes a yellowing of the agar due to the pH change resulting in the colour change of phenol red (Leclercq, 2004; Reissbrodt, 2004; Angelidis *et al.*, 2015). Selective agents in RLM are not known as the formulation for RLM is not publicly available (Angelidis *et al.*, 2015). Although these chromogenic media differ slightly in the way in which they detect pathogenic *Listeria*, they are all acceptable when following ISO 11290-1:2017 and ISO 11290-2:2017 (Gnanou Besse *et al.*, 2019; Rollier *et al.*, 2019).

Previous research has highlighted the potential role that selective and enrichment media may play in the misrepresentation of *L. monocytogenes* lineages, most notably with regard to the selection of lineage II isolates by University of Vermont Medium (UVM) (Jacobsen, 1999; Bruhn *et al.*, 2005; Gorski *et al.*, 2006). Researchers have investigated the role of selective agents, used to inhibit the

growth of background microflora, and their ability to favour the recovery of certain strains of *L. monocytogenes* (Jacobsen, 1999; Beumer and Curtis, 2003; Zilelidou *et al.*, 2016).

Avirulent and hypovirulent *L. monocytogenes* have shown a lower recovery on RLM than virulent strains, suggesting that avirulent strains in particular were susceptible to the selective agents in RLM either causing the repression of virulence-related gene expression or inhibition of enzymatic activity (Gracieux *et al.*, 2003). Previous authors have reported attenuated haemolytic ability of *L. monocytogenes* as a result of the selective agent lithium chloride, and more notably acriflavine and nalidixic acid (Beumer *et al.*, 1997; Jacobsen, 1999; Bruhn *et al.*, 2005; Orsi *et al.*, 2011) and the higher concentration of these agents in BLA (Table 5.4) may be the reason for lower CFU/mL values than the other agars.

Vlaemynck *et al.* (2000) noticed no difference in the recovery of cells between non-selective tryptic soy agar (TSA) and selective ALOA, PALCAM and Oxford agar. Findings from this investigation corroborated this, in that no significant effect (p-value > 0.05) in the CFU/mL on RLM, BLA, HAR and BHI agars was obtained for lineages I and II and different sources (Figure 5.6 & 5.7). It should be noted that the significant effect on CFU/mL for species plated on BHI was expected, as this agar is non-selective, however, it was interesting to notice the significant difference between CFU/mL for *S. sciuri* and *L. monocytogenes* plated together over 24 and 48 hours. The lower counts observed after 48 hours was likely as a result of the larger colonies formed by *S. sciuri*, which masked multiple smaller *L. monocytogenes* colonies.

Table 5.4 Concentration of selective agents in BLA and HAR

Selective agent	g/L	
	Brilliance Listeria Agar	Harlequin™ *Listeria* Chromogenic Agar
Lithium chloride	15.0	10.0
Nalidixic acid	26.0	20.0
Polymyxin B	10.0	10.0
Ceftazidime	6.0	20.0
Amphotericin	10.0	-
Cycloheximide	-	50.0

RLM is a medium that identifies *L. monocytogenes* through the use of PI-PLC function, however it differs from other media that utilise this reaction like ALOA in that phosphatidyl inositol is not used as it can be costly and hard to acquire (Willis *et al.*, 2006). Instead, RLM chromogenic identification of *L. monocytogenes* is through the enzymatic action of PI-PLC on X-inositol phosphate which results in a blue colony. *L. monocytogenes*' inability to ferment xylose allows it to be further differentiated from *L. ivanovii* (Leclercq, 2004), giving it an advantage to HAR and BLA which cannot differentiate *L. ivanovii* as *L. monocytogenes* (Hegde *et al.*, 2007).

One disadvantage to BLA and HAR relying on precipitation zones for the identification of *L. monocytogenes* is the potential growth of non-pathogenic isolate colonies within the precipitation zone of *L. monocytogenes* or *L. ivanovii*. Precipitation zones observed in this study were far more distinct on HAR than on BLA, especially after 24 hours (Figure 5.9). This difference is likely attributed to the differences in differential supplement used as there is no research investigating this. It may also be, as previously mentioned, as a result of attenuated phospholipase expression or inhibition from amphotericin, or the higher concentrations of lithium chloride and nalidixic acid from selective supplements (Table 5.4).

Non-selective BHI also showed a significant effect (p-value ≤ 0.05) on CFU/mL as a result of species (Figure 5.5), indicating a significant difference (p-value ≤ 0.05) in the mixture of *S. sciuri* and *L. monocytogenes* with lower CFU/mL noted after 48 hours. This may be due to *S. sciuri* colony size morphology which is larger than *L. monocytogenes* colonies and may occur in pairs and tetrads (Edman *et al.*, 1968; Kloos *et al.*, 1976), potentially engulfing several smaller *L. monocytogenes* colonies.

The differences observed between the agars could be as a result of the expression of enzymatic activity between strains of *L. monocytogenes* or enzymatic inhibition as a result of selective agents within the media (Beumer *et al.*, 1996, 1997). RLM uses a patented selective solution to ensure inclusive selection of *Listeria* spp. HAR uses lithium chloride as a selective supplement as well as ceftazidime and polymyxin whilst BLA utilises nalidixic acid, lithium chloride, polymyxin and amphotericin (Park *et al.*, 2014). Evidence of lithium chloride inhibiting the growth of *L. monocytogenes* has been previously described (Cox *et al.*, 1990; Jacobsen, 1999), whilst nalidixic acid and polymyxin have shown limited inhibition of *L. monocytogenes* (Jacobsen, 1999; Beumer and Curtis, 2003).

Ultimately findings suggested that all three chromogenic agars were comparable in their ability to culture *L. monocytogenes* from lineages I and II as well as from different sources, except in the case of BLA, which had significantly lower LSM for CFU/mL estimated for lineage II isolates and isolates from the environment (Figure 5.6 and 5.7).

The outcome of the plating of *S. sciuri* yielded meaningful results across the selective media (Figure 5.9). None of the agars indicated *L. monocytogenes*, however, a false-positive for *Listeria* spp. on HAR and BLA was noted, whilst colonies similar to those of *L. innocua* were noted on RLM. Harlequin™ *Listeria* Agar (Neogen, South Africa) completely inhibited the growth of *S. sciuri* where RAPID'L.mono™ agar (Bio-Rad, South Africa) was capable of culturing a limited number of large colonies, and *Brilliance Listeria* Agar (Oxoid, England) was clearly the least effective in selecting against the organism and resulted in the highest enumerability of *S. sciuri*. The role of *S sciuri* in the bacterial community present in the food processing environment as a potential source of resistance and virulence genes, in particular those associated with biofilm formation, make this bacteria a potential contributor to the persistence of *L. monocytogenes* (McLaughlin *et al.*, 2011; Nemeghaire *et al.*, 2014). Contrastingly, there is evidence to support that *S. sciuri* may limit the adhesion and growth of *L. monocytogenes* in biofilms, specifically on stainless steel surfaces, as a result of the production of exopolysaccharide substances (Leriche and Carpentier, 2000).

5.4.2 Plating of inoculated cantaloupe

Cantaloupe has been characterised as providing adequate conditions for the growth of *L. monocytogenes* (Martinez *et al.*, 2016) as it has a high water activity, abundance of natural sugars and a low acid pH of 5.2-6.7 (Harris *et al.*, 2003; Fang *et al.*, 2013; Hong *et al.*, 2014). As with previous findings, no native *L. monocytogenes* was isolated on uninoculated cantaloupe, however, the presence of *B. cereus* capable of growth on RLM was noted. Cantaloupe was chosen due to its historical implication in listeriosis outbreaks.

The effect of multi-strain bacterial mixtures on the detection and enumerability of the three chromogenic agars showed a lower recovery of *L. monocytogenes* ATCC 7644 and *L. monocytogenes* ATCC 19114 and a higher colony count of *L. innocua* ATCC 33091 (data not shown). *L. innocua* has been identified as having the ability to outgrow *L. monocytogenes* when grown in conjunction (Carvalheira *et al.*, 2010).

Findings of the multi-strain bacterial mixture containing both *L. monocytogenes* and *S. sciuri* resulted in both organisms successfully growing on RLM and BLA, however, recovery of *S. sciuri* on HAR was not noted. Furthermore, the occurrence of cantaloupe contaminated with *B. cereus* was only culturable on RLM (Figure 5.9).

5.4.3 Growth of non-*Listeria* isolates

The differences observed between the medias' ability to prevent the growth of non-*Listeria* isolates and select for *L. monocytogenes* could be attributed to several factors, the most immediate being the composition of the agar and the selective agents used (Jacobsen, 1999; Angelidis *et al.*, 2015). In particular the higher concentration of ceftazidime in HAR than in BLA may likely have prevented the growth of *S. sciuri* on HAR as the use of cycloheximide is attributed to the inhibition of yeasts (Vlaemynck *et al.*, 2000). Ha *et al.* (1995) did, however, note that cycloheximide was capable of decreasing the growth rate of *S. aureus*, suggesting that this antibiotic may contribute to the higher selectivity observed by HAR.

Furthermore, the addition of selective supplements to liquid agar at temperatures above those stipulated (46-48^{o}C) may have a significant effect on their ability to inhibit non-*Listeria* isolates, however, there is no published data on the impact of selective supplement performance at temperatures above those recommended by manufacturers.

PI-PLC, one of the principle enzymes relied on for the identification of *L. monocytogenes*, is also produced by *Staphylococcus aureus* (Marques *et al.*, 1989), and *Bacillus cereus* (Otnaess *et al.*, 1977). The production of this virulence factor in non-*Listeria* spp. compromises the specificity of chromogenic methods used for the identification of *L. monocytogenes* potentially masking the organism or resulting in false positives.

Furthermore, evidence of non-*Listeria* growth on ALOA, appearing as blueish-green colonies characteristic of *Listeria* spp. has been previously noted (Angelidis *et al.*, 2015). Similar to this study, Angelidis *et al.* (2015) observed the growth of *S. sciuri*, *Bacillus cereus, Bacillus licheniforms* and *E. faecalis* growth on chromogenic agar (ALOA and RLM). Vlaemynck *et al.* (2000) also noted the growth of *Bacillus* and *Enterococcus* but not *Staphylococcus* spp. with only *Bacillus* showing a vague opaque halo that may allow the bacteria to be mistaken for *L. monocytogenes.*

Willis *et al.* (2006) had similar findings in that the development of non-*Listeria* isolates' colony morphology was slow, and *L. monocytogenes* could only be accurately recognised when plated with contaminant species following 48 hours of incubation and not 24 hours. In this study, clear differences were observed in colonies of isolates after 24 hours on RLM (Figure 5.9). The potential for false negatives from plates with several non-*Listeria* appearing as *Listeria* spp. on HAR and BLA may mask any *Listeria* spp. that may have been cultured in low numbers (Angelidis *et al.*, 2015).

5.4.3.1 *Staphylococcus sciuri*

Findings from this study indicate the ability of *Staphylococcus sciuri* to grow on selective media. *S. sciuri* is a Gram-positive, facultatively anaerobic cocci shaped bacteria which is non-motile and non-spore forming (Kloos *et al.*, 1976). It is typically associated with animals, however, it is increasingly seen amongst human clinical cases (Dakić *et al.*, 2005). *S. sciuri* can be isolated from the environment where it is found in both soil and water sources and can grow at temperatures of 15 ^{o}C (Kloos *et al.*, 1976; Kloos, 1980). It is more frequently isolated from animal products than from fresh produce and has a number of potential virulence factors (Stepanović *et al.*, 2001). *S. sciuri* has also been well documented as a reservoir for antimicrobial resistance determinants (Dakić *et al.*, 2005; Nemeghaire *et al.*, 2014). More concerning is evidence of the ability to transfer resistance determinants with other bacteria (Wu *et al.*, 1998), and what influence this may potentially have on *L. monocytogenes* in the food industry.

The association of *L. monocytogenes* with *S. sciuri* raises concerns regarding the interspecies interactions of these two organisms and what role *S. sciuri* may potentially play regarding the survivability of *L. monocytogenes* in the prepared-fruit industry. *S. sciuri* is capable of effectively forming biofilms, especially under nutrient-limited conditions (Stepanović *et al.*, 2003). This is advantageous to *L. monocytogenes* as it offers the opportunity for the transfer of genetic material and increased protection from disinfectants and cleaning practices (Valderrama and Cutter, 2013; Carpentier, 2014). *S. sciuri* biofilm forming ability is, however, less than some other *Staphylococcal* spp. (Marino *et al.*, 2011).

HAR showed a higher selectivity than RLM or BLA, inhibiting the growth of all *S. sciuri*. *S. sciuri* growth on RLM and non-selective BHI agar resulted in colony morphology typical of previous descriptions (Kloos *et al.*, 1976; Angelidis *et al.*, 2015). Large, raised colonies with a cream tint were observed (Figure 5.9). These colonies can be similar to those of *L. innocua*, however, when compared there are distinguishable differences between the bacteria on RLM (Figure 5.9). The potential for the misidentification of *L. innocua* from *S. sciuri* is a concern for the environmental monitoring of *Listeria* spp. in factories, more so the similarity in colony morphology observed on BLA as formation of the blue/green colour is a determining characteristic for *Listeria* spp. on this agar, with very few differences noted between *L. innocua* and *S. sciuri* (Figure 5.9). BLA supported the growth of all four *S. sciuri* and resulted in a greater number of colonies than RLM. This is of concern, as presumptive false-positives of *S. sciuri* may lead to more costly and unnecessary *Listeria* management decisions. It should be noted that *S. sciuri* has previously shown an ability to negatively influence the ability of *L. monocytogenes* to adhere to surfaces and form biofilms. This may contribute to mitigating build-up of the pathogen in between cleaning (Leriche and Carpentier, 2000).

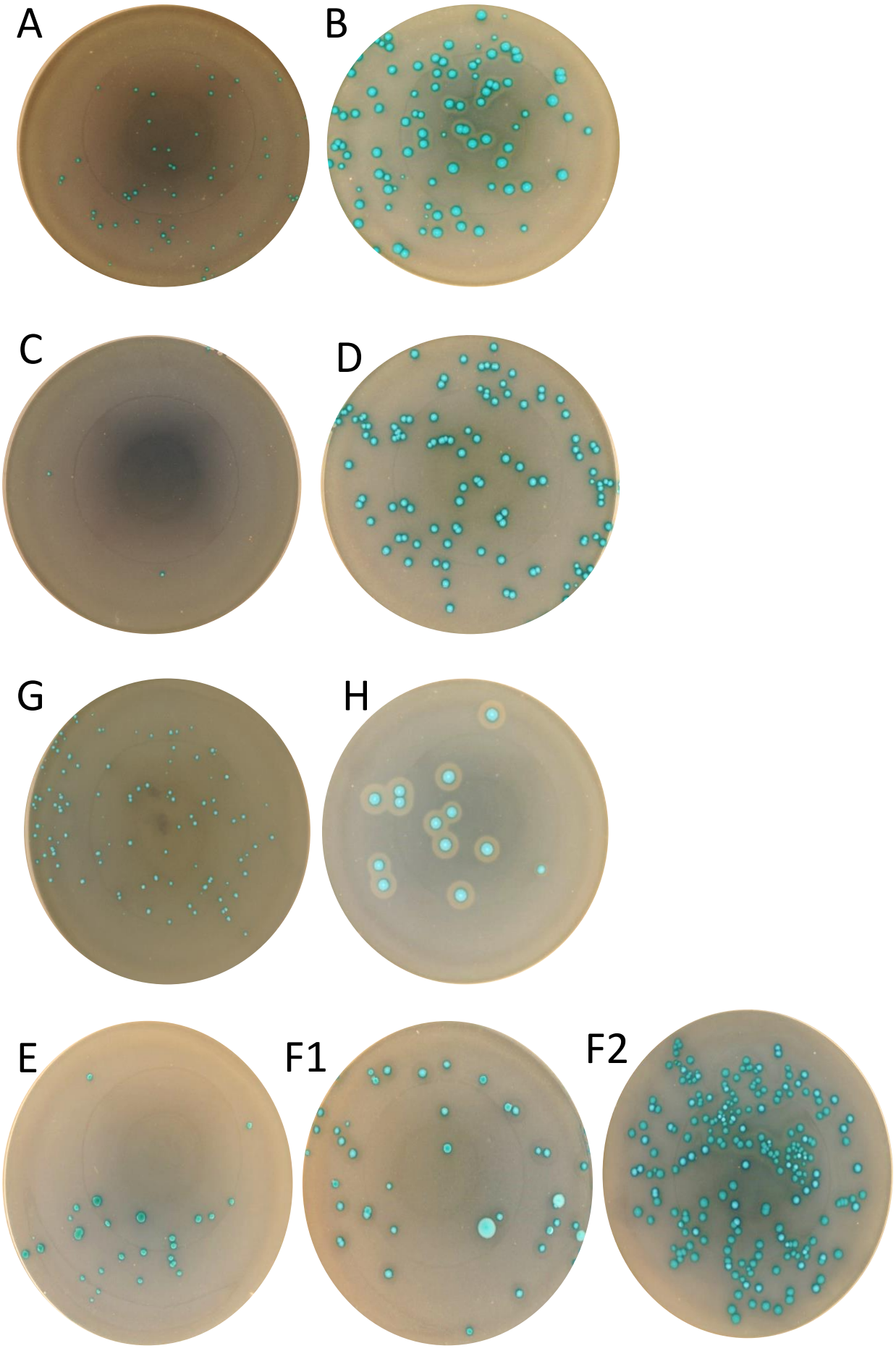
5.4.3.2 *Bacillus cereus*

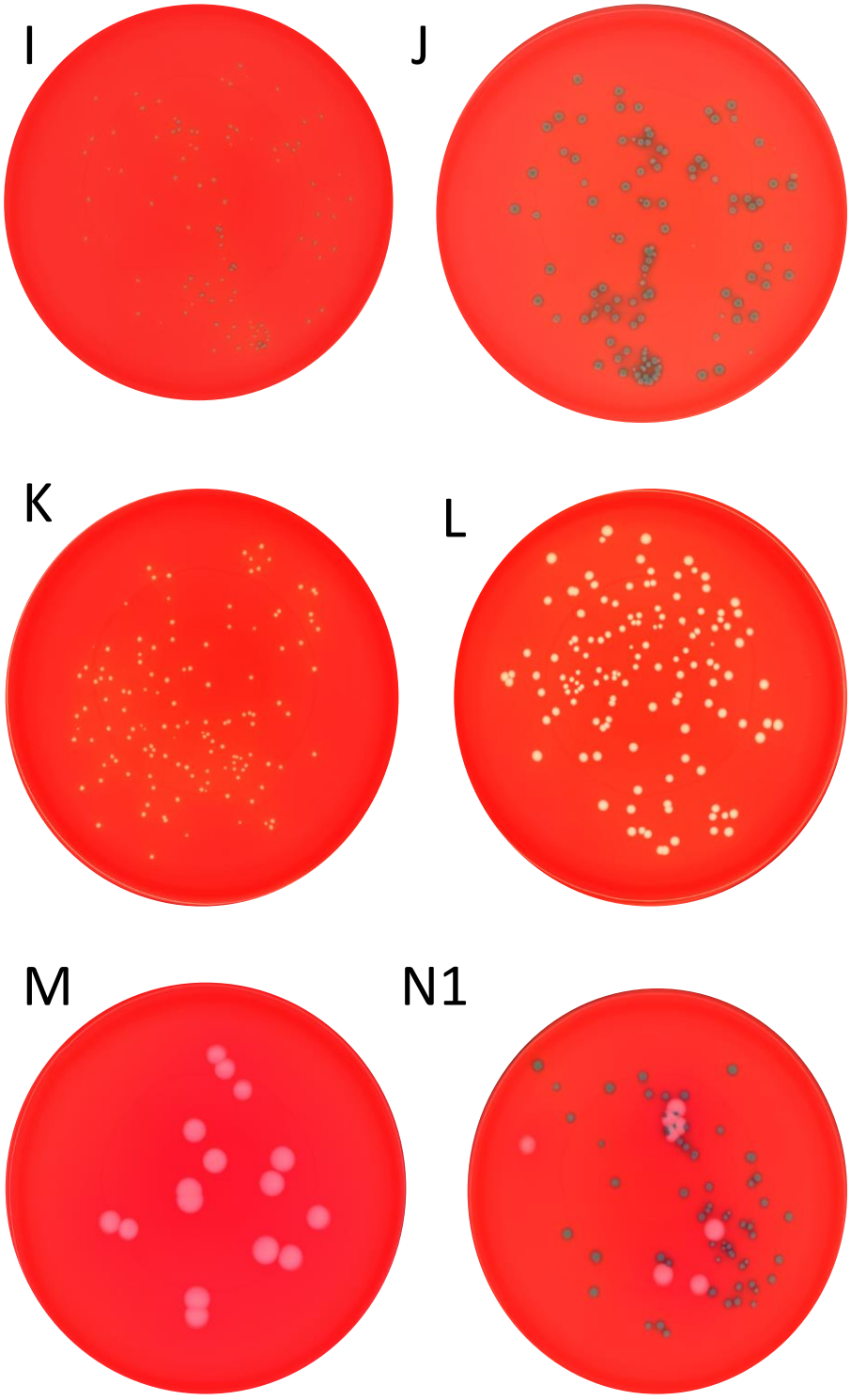
The spiking of cantaloupe to determine the effect of native microflora of a *Cucumis melo*, a fruit vector for *L. monocytogenes*, revealed the growth of uncharacteristic colonies on RLM but not on HAR or BLA. These were subsequently determined to be *Bacillus cereus*. The growth of this bacteria on RLM has been observed previously. Its ability to produce phospholipases PI-PLC and PC-PLC, both virulence-associated products utilised in the detection of *L. monocytogenes* on chromogenic agar, as well as β-D-glucosidase production enables the formation of blue/green colonies (Vlaemynck *et al.*, 2000; Willis *et al.*, 2006; Angelidis *et al.*, 2015). This makes the potential misidentification of this organism as pathogenic *Listeria* possible. Its growth on RLM was, however, uncharacteristic of pathogenic *Listeria* (Figure 5.9). Although these colonies were not similar enough to *L. monocytogenes* to cause misidentification, their ability to grow on selective media is cause for concern due to the potential for false positives.

5.4.3.3 *Enterococcus faecalis*

The occurrence of *Enterococcus faecalis* growth on RLM was surprising. This organism is used as a negative control for BLA. Its occurrence in chromogenic selective agar is not uncommon. This organism is capable of esculinase activity and has previously been noted as a common contaminant on selective agar like PALCAM and Oxford agar (Restaino *et al.*, 1999).

Da Silva Fernandes *et al.* (2015) observed better biofilm forming ability by *L. monocytogenes* in mixed species biofilms with *Enterococcus* spp. at ambient temperatures of 25^{o}C. This raises concerns regarding the potential this organism has to mask *L. monocytogenes* and create false-positive results.





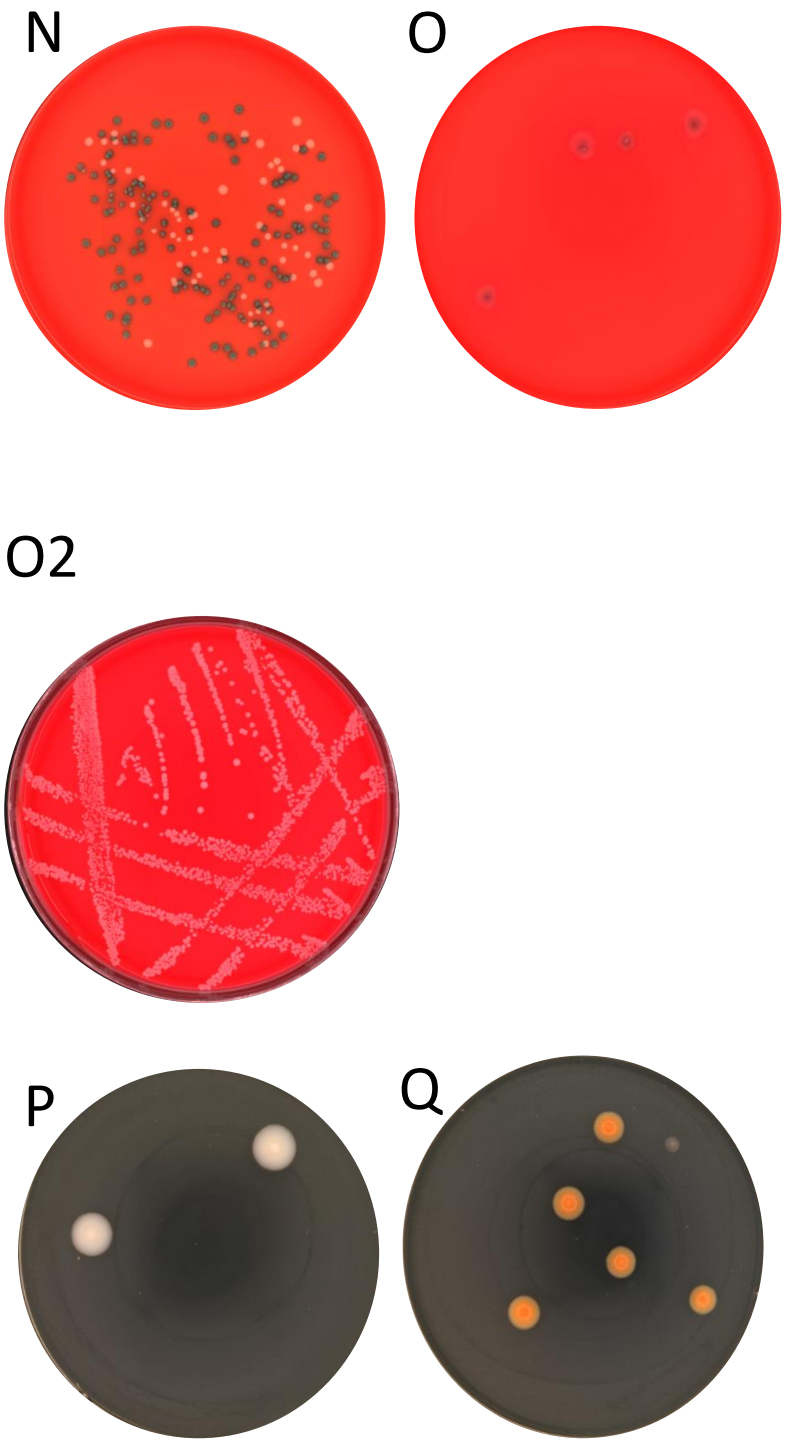


Figure 5.9 Pictures showing the appearance of colonies on the four media used in this study. A – *L. monocytogenes* (LM) on BLA after 24 hours; B – LM on BLA after 48 hours; C – *L. innocua* (LI) on BLA after 24 hours; D – LI on BLA after 48 hours; E – *S. sciuri* (SS) on BLA after 48 hours; F1 – SS and LM on BLA after 48 hours; F2 – LI and LM on BLA after 48 hours; G – LM on HAR after 24 hours; H – LM on HAR after 48 hours; I – LM on RLM after 24 hours; J – LM on RLM after 48 hours; K – LI on RLM after 24 hours; L – LI on RLM after 48 hours; M – SS on RLM after 48 hours; N1 – SS and LM on RLM after 48 hours; N2 – LI and LM on RLM after 48 hours; O – *B. cereus* on RLM after 48 hours; O2 – *E. faecalis* on RLM after 48 hours; P and Q – SS on BHI after 48 hours.

5.5 Conclusion

These findings conclude that in an analysis of *L. monocytogenes* on chromogenic agar, all three media yielded comparable findings. BLA was the only media not to show a significant difference in CFU/mL with the plating of *S. sciuri*, *L. monocytogenes* and a mixture of the *L. monocytogenes* and *S. sciuri*. BLA also showed a significant difference in the LSM between lineage II isolates plated onto the media in comparison to lineage II isolates plated onto RLM and BHI. BLA also indicated significant difference in the LSM of isolates from the environment compared to the other three agars, in all cases estimating lower CFU/mL. It should not be overlooked that the ability for *S. sciuri* isolates to grow on RLM and BLA is a major drawback to ensuring false positives do not occur and that *L. monocytogenes* is not overlooked. Furthermore, the growth of *B. cereus* group and *E. faecalis* on RLM suggests that this media may require a greater selectivity and that lab technicians should have a good understanding of *L. monocytogenes* colony morphology on this media. Thus, all colonies should be regarded as presumptive *L. monocytogenes* and should be confirmed using other more discriminatory methods.

5.6 References

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Chapter 6: General discussion and conclusion

6.1 Conclusion

L. monocytogenes is a robust pathogen that remains of concern in ensuring the safety of RTE food products. Management of this pathogen within the prepared-fruit industry to ensure food safety is of vital importance as consumer trends move towards healthy diets high in fruit and vegetables (Rico *et al.*, 2007; Brunner *et al.*, 2010; Ronquest-Ross *et al.*, 2015). Understanding the relationship between this pathogen and the RTE prepared-fruit processing facility is crucial to developing strategies to mitigate the organism's survival and proliferation. Recent listeriosis outbreaks linked to fruit include a 2011 outbreak in the USA, linked to whole cantaloupe, as well as, an outbreak in 2018 in Australia linked to rockmelon (McCollum *et al.*, 2013; Das, 2019). Furthermore, South Africa fell victim to the largest listeriosis outbreak recorded, when contaminated deli-meat caused more than 1000 infections and 200 deaths (Tambo *et al.*, 2018; Thomas *et al.*, 2020).

RTE prepared-fruit is not considered a high-risk product for *L. monocytogenes*, however, recent instances of fruit-related *L. monocytogenes* outbreaks is cause for investigation. This thesis aimed to assess the role of the agricultural environment and packhouse in contamination of cantaloupe and watermelons pre-harvest, the sources of *L. monocytogenes* contamination within the prepared-fruit processing environment, as well as the distribution of genetic lineages. Findings indicated that the sampling of melon growers in Limpopo and the Eastern Cape revealed vastly different results. Swabs taken at three melon farms in Limpopo during spring did not detect any *Listeria* spp., whilst swabs taken at two farms in the Eastern Cape during autumn indicated a range of *Listeia* spp. including *L. monocytogenes*. Rainfall and lower temperatures in the Eastern were likely the cause for the differences with Limpopo, which was hotter and where no rainfall was experienced. This supported findings which indicated that cooler wetter weather was shown to increase the likelihood of *L. monocytogenes* contamination from the soil as well as being advantageous to its ability to compete with other microbiota (Ivanek *et al.*, 2007; Linke *et al.*, 2014; Chersich *et al.*, 2018). The significance of understanding the role of certain weather events or conditions on the extent of *Listeria* contamination allows RTE prepared-fruit processors to increase the level of hazard prevention and control accordingly, in order to decrease the risks of contamination.

The assessment of *L. monocytogenes* isolated from the RTE prepared-fruit processing facility was aimed at understanding and investigating the distribution of the organism and the different genetic lineages in this enigmatic environment. *L. monocytogenes* was isolated from a range of raw materials

(cantaloupe, watermelon, papaya, avocado, guava) as well as from the environment (knives, chopping boards, peelers, drains, crates) and in final products (avocado pulp, melon salad). PCR-RFLP lineage typing allowed the differentiation of *L. monocytogenes* isolates and revealed the occurrence of both lineage I and lineage II, with the majority of *L. monocytogenes* belonging to lineage II, and a limited number of lineage I isolates most prevalent in final product samples. The abundance of lineage II samples within the environment has been previously noted, however, final products harbouring lineage I isolates – associated with clinical cases of listeriosis and implicated in a number of outbreaks – was of concern and was an indication of the potential vector that prepared-fruit product can play for this deadly pathogen (Orsi *et al.*, 2011).

In order to investigate the effectiveness of sanitation procedures and assess new solutions to eliminate *L. monocytogenes* within the industrial prepared-fruit environment, sanitisers normally employed by the facility (peracetic acid, chlorine and Byotrol™ QFS) were assessed for their efficacy with significant effects caused by source or lineage determined through a repeated measures ANOVA. It was found that both chlorine and peracetic acid resistance amongst isolates was prevalent, however Byotrol QFS showed efficacy against all isolates with no evidence of resistance. The occurrence of peracetic acid and chlorine resistance in *L. monocytogenes* isolates located in the prepared-fruit processing facility indicates the potential for the persistence of these organisms. Peracetic acid and chlorine resistance may occur as a result of sub-lethal exposure caused by improper cleaning prior to sanitising or improper application of sanitisers, as well as overuse and the potential selective pressure this may cause (Parish *et al.*, 2003; Allen *et al.*, 2016; Singh *et al.*, 2018; Fagerlund *et al.*, 2020). The use of peracetic acid and chlorine in the agricultural environment and packhouse may also contribute to resistance in organisms within the processing facility. Application of these sanitisers on whole fruit which are normally covered in debris, or may have rough surfaces that prevent adequate surface contact of the sanitisers, can result in resistant strains within the industrial prepared-fruit processing facility. The results acquired indicated no significant difference (p-value > 0.05) in sanitiser resistance amongst lineages I and II, as well as no significant difference between the sources of isolates. This indicates that amongst the isolates from the prepared-fruit processing facility, there was no effect on sanitiser resistance due to the lineage or source of isolates, even though previous researchers have indicated higher survivability and sanitiser resistance amongst lineage II isolates, as well as from previous stress adaptations resulting from an isolates source. Both chlorine and peracetic acid should not be solely trusted to eliminate *L. monocytogenes* but should be used as a means of reducing the number of cells; whilst Byotrol QFS was effective in eliminating all isolates and should be the preferred sanitiser for the management of *L. monocytogenes*, although rotation of sanitisers is encouraged to prevent the persistence and growth of any resistant strains.

The role of chromogenic agar in providing rapid results detecting contamination in raw materials and food products, as well as assessing the effectiveness of cleaning and sanitation practices, is paramount to ensuring food safety and assessing *L. monocytogenes* risk mitigation practices. Three chromogenic agars commonly employed for the detection of *L. monocytogenes* and accepted in the use of ISO 11290 and ISO 11290-2:2017 were compared. These media were assessed to determine differences in their selectivity, enumerability and detection of *L. monocytogenes* and the common environmental contaminant *S. sciuri* and common microbiota present on cantaloupe. The role of lineage type and source was also investigated to determine lineage-based culturability bias, as well as the impact of stresses related to an isolates source. Findings indicated that all three media achieved comparable results, however, both RAPID'L.mono™ media and *Brilliance Listeria* Agar lacked the ability to inhibit the growth of *S. sciuri*, with RAPID'L.mono™ further supporting both *E. faecalis* (used as a negative control) and *B. cereus* group (present on store-bought cantaloupe). The growth of these contaminating bacteria on chromogenic agar has been noted in previous research studies (Leriche and Carpentier, 2000; Angelidis *et al.*, 2015). *Brilliance Listeria* Agar was also the only chromogenic media which uncharacteristically indicated no significant difference in the CFU/mL of *S. sciuri, L. monocytogenes* and a combination of the two. Furthermore, *Brilliance Listeria* Agar had significantly lower (p-value ≤ 0.05) CFU/mL for lineage II isolates in comparison to RAPID'L.mono™ and non-selective Brain Heart Infusion agar. This was also the case with BLA indicating significantly lower estimates of CFU/mL for isolates from the environment in comparison to the other three media. In terms of selectivity, HAR presented the best results, whilst RLM most supported the growth of non-*Listeria* bacteria, although the morphology of these colonies was distinguishable from those of presumptive *L. monocytogenes*. The role of *S. sciuri* growth is concerning in that this organism may potentially mask the presence of *Listeria* spp., as seen in significantly lower CFU/mL estimates for a mixture of *S. sciuri* and *L. monocytogenes* after 48 hours in comparison to 24 hours when cultured on non-selective BHI agar. The potential misrepresentation of non-*Listeria* species on chromogenic agar is something which lab technicians should be aware of when using RLM and BLA. Furthermore, the potential of BLA to significantly underestimate CFU/mL for isolates from the environment should be taken into consideration during testing for enumerability. Findings also indicated no significant effect in the recovery of isolates on the different media due to lineage type. Although RLM lacked the level of selectivity of the other two chromogenic media, the ability of this media to differentiate between the two pathogenic *Listeria* spp. (*L. monocytogenes* and *L. ivanovii*) makes this media invaluable in its ability to provide presumptive classification down to the species level.

The results obtained indicate the occurrence of the human pathogen *L. monocytogenes* in the ready-to-eat prepared-fruit supply chain, from agricultural environment and packhouse, up to the industrial

prepared-fruit processing facility. The prevalence of *Listeria* spp. pre-harvest was shown to be exacerbated by weather, whilst occurrence of *L. monocytogenes* within the processing facility was predominantly on raw materials and within the environment and was dominated by lineage II isolates, whilst lineage I isolates were prevalent in avocado pulp final product. Chlorine and peracetic acid resistance amongst isolates is concerning as these sanitisers are a major tool for the control of the pathogen in ready-to-eat prepared-fruit products, which cannot undergo heat sterilisation due to the negative impacts on the quality of these products. Control of *L. monocytogenes* could be improved through better implementation of cleaning protocols to reduce the potential for sub-lethal exposure of peracetic acid and chlorine amongst *L. monocytogenes* populations and by using the highly effective Byotrol QFS. Effective monitoring of *L. monocytogenes* was shown to be achieved comparably between the three chromogenic media assessed, with only Harlequin™ *Listeria* Chromogenic Agar capable of inhibiting the growth of all non-*Listeria* isolates present, and with *Brilliance Listeria* Agar (Oxoid) being the only media to indicate an effect on CFU/mL due to source and lineage in comparison to the other media. RLM supported the growth of a number of non-*Listeria* isolates but is capable of differentiating between *L. monocytogenes* and *L. ivanovii*.

The ready-to-eat prepared-fruit industry is dynamic and requires responsive and tailored solutions for the management of *L. monocytogenes*. Understanding the effects of weather on pre-harvest contamination can allow processors to take appropriate action to reduce the levels of contamination, whilst understanding the propensity of the organism to occur on the surface of a wide range of fruit can help prevent contamination of fruit with a mildly acidic pH that can support the growth of the organism. The need to assess sanitiser-resistance levels among isolates should also form part of *L. monocytogenes* risk mitigation, as this study identified resistance to two of the three sanitisers regularly used to eliminate this pathogen. Finally, the monitoring and testing of *L. monocytogenes* using chromogenic media should be complimented with training of lab technicians to understand colony morphology and the propensity for false-positives and negatives or the masking of pathogenic species by background microbiota. *L. monocytogenes* in the ready-to-eat prepared-fruit industry remains poorly characterised, further research to understand the most appropriate and effective means for the industry to better manage this organism would be hugely beneficial especially as this category of food product remains considered low-risk for *L. monocytogenes* contamination. Larger sample sets of several hundred and investigations that assess solutions beyond those currently employed – like novel disinfection technology such as sanitisers and SHS treatment would greatly contribute to the understanding of this organism within this industry and contribute to solutions that will prevent the potential for future outbreaks of listeriosis.

6.2 References

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